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(54) Title: METHODS FOR INHIBITING ANTIGEN SPECIFIC T CELL RESPONSES

(57) Abstract

Methods for inhibiting antigen-specific T cell responses by use of an agent which inhibits a costimulatory signal in T cells are disclosed. Preferably, both a first agent which inhibits a costimulatory signal in the T cell (e.g., a CTLA4Ig fusion protein) and a second agent which inhibits another T cell function, such as adhesion of the T cell to a cell presenting antigen to the T cell, are used to inhibit antigen-specific T cell responses. For example, to inhibit adhesion of a T cell to a cell presenting antigen, an anti-LFA-1 antibody can be used in conjunction with a CTLA4Ig fusion protein. Alternatively, another agent which inhibits a costimulatory signal in T cells, such as an anti-B7-1 antibody or an anti-B7-2 antibody can be used with a second agent which inhibits a proliferative signal in the T cell e.g., an anti-IL-2 receptor antibody. The methods of the invention are particularly useful for inhibiting graft versus host disease and for inhibiting rejection of a transplanted tissue or organ.

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METHODS FOR INHIBITING ANTIGEN SPECIFIC T CELL RESPONSES

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Background of the Invention

Allogeneic bone marrow transplantation (BMT) is an effective treatment for many hematological malignancies and severe aplastic anemia (see e.g., Thomas, E.D. (1983) *J. Clin. Oncol.* 1:517-531; O'Reilly, R.J. et al. (1983) *Blood* 62:942-964; and Storb, T. et al. *Semin. Hematol.* 2:27-34). However, the alloreactivity of T cells within the donor bone marrow to recipient cells leads to a potentially fatal condition referred to as graft versus host disease (GVHD). One therapeutic approach which has been taken in an attempt to minimize or eliminate GVHD involves administration to the transplant recipient of a general immunosuppressant, such as cyclosporin A or methotrexate (see e.g., Kapoor, N. et al. (1989) *Bone Marrow Transplant.* 4:153). Use of such agents, however, is associated with deleterious side effects, including kidney damage and an increased susceptibility to infections. Another approach taken to minimize or eliminate GVHD has been to deplete donor bone marrow of T cells in an attempt to remove alloreactive T cells (see e.g., Martin, P.J. et al. (1987) *Adv. Immunol.* 40:379). While T cell depletion has been found to reduce the occurrence of GVHD, this treatment also reduces the success of bone marrow engraftment. Additionally, depletion of T cells from donor bone marrow used to treat hematological malignancies reduces the anti-leukemic activity (also referred to as the graft versus leukemia response, or GVL) of the donor cells (see e.g., Goldman, J.M. et al. (1988) *Ann. Intern. Med.* 108:806-814; Marmont, A.M. et al. (1991) *Blood* 78:2120-2130). Thus, while the presence of alloreactive T cells within a bone marrow graft has the detrimental effect of inducing GVHD, the presence of at least some T cells within the graft is beneficial both for successful engraftment and for anti-leukemic responses. A therapy that effectively inhibits the responses of alloreactive T cells within donor bone marrow while permitting the continued presence and function of other T cells within the graft would therefore be of great advantage in the addressing the problem of GVHD while promoting the efficacy of bone marrow engraftment.

The induction of a T cell response has been shown to require two signals: a first signal provided by stimulation through the antigen-specific T cell receptor (TCR) on the surface of the T cell, and a second signal (termed a costimulatory signal) provided by ligation of one or more other T cell surface receptors. Engagement of the TCR alone (i.e., signal 1) in the absence of a costimulatory signal (i.e., signal 2) induces a state of unresponsiveness, or anergy, in the T cell. A costimulatory signal can be generated in a T cell by stimulation of

the T cell through a cell surface receptor CD28 (Harding, F. A. (1992) *Nature* 356:607-609). Ligands for CD28 have been identified on antigen presenting cells (APCs). CD28 ligands include members of the B7 family of proteins, such as B7-1(CD80) and B7-2 (CD86) (Freedman, A.S. et al. (1987) *J. Immunol.* 137:3260-3267; Freeman, G.J. et al. (1989) *J. Immunol.* 143:2714-2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174:625-631; Freeman, G.J. et al. (1993) *Science* 262:909-911; Azuma, M. et al. (1993) *Nature* 366:76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178:2185-2192). Additionally, B7 family members have been shown to bind another surface receptor on T cells related to CD28 termed CTLA4 (Linsley, P.S. (1991) *J. Exp. Med.* 174:561-569; Freeman, G.J. et al. (1993) *Science* 262:909-911).

The characterization of the receptors and ligands involved in T cell costimulation has led to therapeutic approaches based upon induction of antigen specific T cell unresponsiveness by blocking of a costimulatory signal in T cells. For example, a CTLA4Ig fusion protein, which binds both B7-1 and B7-2, has been used to inhibit rejection of cardiac allografts and pancreatic islet xenografts (see e.g., Turka, L.A. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 11102-11105; Lin, H. et al. (1993) *J. Exp. Med.* 178:1801-1806; Lenschow, D.J. et al. (1992) *Science* 257, 789-792). Similarly, antibodies reactive with B7-1 and/or B7-2 have been used to inhibit T cell proliferation and IL-2 production *in vitro* and inhibit primary immune responses to antigen *in vivo* (Hathcock K.S. et al. (1993) *Science* 262, 905-907; Azuma, M. et al. (1993) *Nature* 366:76-79; Powers, G.D. et al. (1994) *Cell. Immunol.* 153, 298-311; Chen C. et al. (1994) *J. Immunol.* 152, 2105-2114). However, effective methods for inhibiting T cell responses in transplant situations which avoid the need for general immunosuppression of the transplant recipient and overcomes the drawbacks of T cell depletion in bone marrow transplants are still needed and would have widespread therapeutic applications.

Summary of the Invention

This invention features improved methods for inhibiting a T cell response to an antigen by use of at least one agent which inhibits a costimulatory signal in the T cell. This invention is based, at least in part, on the discovery that an inhibitor of a costimulatory signal in T cells can be used *in vitro* or *in vivo* to inhibit inappropriate T cell responses to antigen in clinical situations, such as bone marrow and organ transplantation, as well as autoimmune disorders and allergic responses. The inhibitor of a costimulatory signal in T cells is preferably an agent which inhibits an an interaction between a receptor on the T cell (e.g., CD28 and/or CTLA4) and a costimulatory molecule (e.g., B7-1 and/or B7-2) on a cell presenting antigen to the T cell. Thus, the inhibitor of a costimulatory signal can be, for example, an antibody (or fragment thereof) which binds the receptor or the costimulatory molecule, a soluble form of the receptor or costimulatory molecule or a peptide fragment or other small molecule designed to inhibit a costimulatory signal in T cells. A preferred

inhibitor is a soluble CTLA4-immunoglobulin fusion protein (CTLA4Ig) or an anti-B7-1 antibody or an anti-B7-2 antibody (or both an anti-B7-1 and an anti-B7-2 antibody).

According to the methods of the invention, a T cell response is inhibited by contacting the T cell with at least one inhibitor of a costimulatory signal in an antigen specific T cell. In particular, for use in inhibiting graft versus host disease in a bone marrow transplant recipient, an agent such as a soluble form or CTLA4 or an anti-B7-1 or anti-B7-2 antibody can be used to treat the donor bone marrow *in vitro*, to thereby inhibit donor T cell responses to cells expressing recipient alloantigens, prior to administration of the bone marrow to the recipient.

In another aspect of the invention, an inhibitor of a costimulatory signal in T cells (or first agent) is used in conjunction with at least one second agent which, when combined with the first agent, inhibits inappropriate T cell responses to antigen in bone marrow or organ transplantation, as well as autoimmune disorders and allergic responses. In one embodiment, the second agent inhibits adhesion of the T cell to a cell presenting antigen to the T cell. For example, the second agent can act to inhibit an interaction between an adhesion molecule on the T cell and a ligand for the adhesion molecule on a cell presenting antigen to the T cell. Suitable adhesion molecule and ligands to be targeted for inhibition include LFA-1, ICAM-1, ICAM-2, ICAM-3, VLA-4, VCAM-1, LECAM-1, ELAM-1, and CD44. Antibodies (or fragments thereof) that bind the adhesion molecule or receptor, or soluble forms of the adhesion molecule or receptor, can be used as the second agent in the methods of the invention. A preferred second agent is an anti-LFA-1 antibody.

In another embodiment of the invention, an inhibitor of a costimulatory signal in T cells is used with a second agent which inhibits generation of a proliferative signal in the T cell, to thereby inhibit T cell responses to antigen. For example, an agent which inhibits an interaction between a receptor on the T cell and a T cell growth factor, such as interleukin-2 or interleukin-4 can be used with, for example, a soluble form of CTLA4. Thus, the second agent can be an antibody (or fragment thereof) which binds either the receptor on the T cell or the T cell growth factor. A preferred second agent for use in the method of the invention is an anti-interleukin-2 receptor (IL-2R) antibody (or fragment thereof). Alternatively, the second agent may act intracellularly to inhibit a proliferative signal in the T cell.

The the above-described agents are particularly useful for inhibiting graft versus host disease in a bone marrow transplant recipient in which a first agent which inhibits generation of a costimulatory signal in a donor T cell (e.g., CTLA4Ig) and a second agent which inhibits adhesion of a donor T cell to a cell presenting antigen to the T cell (e.g., an anti-LFA-1 antibody) are administered to a bone marrow transplant recipient to inhibit donor T cell responses to cells expressing recipient alloantigens. Alternatively, a second agent which inhibits generation of a proliferative signal in a donor T cell (e.g., an anti-IL-2R antibody) can be administered to the recipient in conjunction with the first agent. In addition to administering the first and second agents to the recipient, donor cells (e.g., donor bone

marrow) can be contacted with the first and second agents *in vitro* in the presence of recipient cells prior to transplantation of the donor cells into the recipient. Preferably, the donor cells are first cultured with recipient cells *in vitro* as a priming step prior to being contacted with the first and second agents. This regimen, involving priming of donor cells to recipient antigens *in vitro*, followed by exposure of the primed donor cells to the first and second agents *in vitro* in the presence of recipient cells, followed by administration of the donor cells to the recipient along with continued treatment of the recipient *in vivo* with first and second agents, has been found to be particularly effective in inhibiting GVHD in a bone marrow transplant recipient. Alternatively, donor cells can be contacted with the first and second agents *in vitro* in the presence of recipient cells (preferably following preculture with recipient cells) and then administered to the recipient without further *in vivo* treatment of the recipient with the first and second agents.

The methods of the invention are also useful for inhibiting rejection of other types of grafts (e.g., organ or tissue grafts such as heart, kidney, liver, lung, skin, pancreatic islets, etc.) in a transplant recipient. Accordingly, the first and second agents described above can be administered to an organ or tissue transplant recipient. In addition to administering the first and second agents, donor cells (such as hematopoietic cells) can also be administered to the recipient as a priming step prior to transplantation of the graft.

Pharmaceutical compositions suitable for administration are also within the scope of the invention. In one embodiment, the composition comprises an amount of a human CTLA4-immunoglobulin fusion protein or an anti-human B7-1 or B7-2 antibody (or both an anti-human B7-1 and B7-2 antibody) and an amount of an anti-human LFA-1 antibody in a pharmaceutically acceptable carrier. In another embodiment, the composition comprises an amount of a human CTLA4-immunoglobulin fusion protein or an anti-human B7-1 or B7-2 antibody (or both an anti-human B7-1 and B7-2 antibody) and an amount of an anti-human interleukin-2 receptor antibody in a pharmaceutically acceptable carrier.

Another aspect of the invention features novel bispecific molecules having a first binding specificity for a costimulatory molecule (e.g., B7-1, B7-2, B7-3) or costimulatory receptor (e.g., CTLA4, CD28) and a second binding specificity for an adhesion molecule (e.g., LFA-1, ICAM-1, ICAM-2, ICAM-3) or growth factor receptor (e.g., IL-2R). Such molecules can be used in the methods as described herein.

Brief Description of the Drawings

Figure 1 is a graphic representation of the percent survival of bone marrow transplant (BMT) recipient mice treated with either phosphate buffered saline (PBS) (control), CTLA4Ig alone, CTLA4Ig and anti-IL-2R or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen.

Figure 2 is a graphic representation of the mean weight in grams of BMT recipient mice treated with either PBS (control), CTLA4Ig alone, CTLA4Ig and anti-IL-2R or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen.

Figure 3 is a graphic representation of the percent survival of BMT recipient mice treated with either PBS (control), CTLA4Ig alone, anti-LFA-1 alone, or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, or treated with CTLA4Ig and anti-LFA-1 using an *in vivo* treatment regimen alone.

Figure 4 is a graphic representation of the mean weight in grams of BMT recipient mice treated with either PBS (control), CTLA4Ig alone, anti-LFA-1 alone, or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, or treated with CTLA4Ig and anti-LFA-1 using an *in vivo* treatment regimen alone.

Figure 5 is a graphic representation of the percent survival of BMT recipient mice treated with either PBS (control) or CTLA4IG + anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, wherein donor cells were either primed or not primed with recipient cells prior to *in vitro* treatment.

Figure 6 is a graphic representation of the mean weight in grams of BMT recipient mice treated with either PBS (control) or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, wherein donor cells were either primed or not primed with recipient cells prior to *in vitro* treatment.

Figure 7 is a graphic representation of the percent survival of BMT recipient mice treated with either PBS (control), CTLA4Ig alone, CTLA4Ig and anti-IL-2R or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, or treated with CTLA4Ig and anti-IL-2R *in vitro* and CTLA4Ig alone *in vivo*.

Figure 8 is a graphic representation of the mean weight in grams of BMT recipient mice treated with either PBS (control), CTLA4Ig alone, CTLA4Ig and anti-IL-2R or CTLA4Ig and anti-LFA-1, using a combined *in vitro* and *in vivo* treatment regimen, or treated with CTLA4Ig and anti-IL-2R *in vitro* and CTLA4Ig alone *in vivo*.

Figure 9 is a graphic representation of the percent survival of BMT recipient mice which received either untreated donor cells (control) or donor cells treated *in vitro* with either CTLA4Ig alone or paraformaldehyde fixed recipient cells.

Figure 10 is a graphic representation of the percent survival of alloreactive T cell recipient mice treated with either PBS (control), anti-B7-1 antibody, anti-B7-2 antibody, a combination of anti-B7-1 and anti-B7-2 antibodies, or CTLA4Ig.

Figure 11 is a graphic representation of the percent survival of alloreactive T cell recipient mice treated with either PBS (control), anti-IFN-γ, anti-IL12, or with a combination of anti-B7-1 and anti-B7-2 antibodies.

Figure 12 is a graphic representation of the percent survival of alloreactive T cell recipient mice treated with either PBS (control), a combination of anti-B7-1 and anti-B7-2 antibodies, hCTLA4Ig, Rapamycin, mIL-10, IL-12, a combination of anti-CD2 and anti-CD48 antibodies, or anti-gp39 antibody.

Figure 13 is a graphic representation of the percent survival of BMT recipient mice treated with anti-ICAM-2 antibody, anti-gp39 or an anti-LFA-1 antibody, or in which the BMT was first depleted of NK cells by treatment with anti-NK1.1 antibody and complement (anti-NK1.1 + complement).

Detailed Description of the Invention

This invention features methods for inhibiting antigen specific T cell responses *in vitro* and/or *in vivo* by use of at least one agent which inhibits a costimulatory signal in T cells alone, or in combination with a second agent which either inhibits adhesion of the T cell to a cell presenting antigen to the T cell or inhibits generation of a proliferative signal in the T cell. As used herein, the phrase "inhibiting or inhibition of a T cell response" refers to a reduction in or substantial elimination of at least one T cell response, such as T cell proliferation, lymphokine secretion or induction of an effector function (e.g., induction of cytotoxic T cell activity or antibody production by B cells), upon exposure of the T cell to an antigen. The phrase "inhibiting or inhibition of a T cell response" is intended to encompass suppression of the response of a T cell to an antigen as well as induction of unresponsive in the T cell to the antigen, also referred to herein as induction of anergy in the T cell. A T cell which has been rendered unresponsive, or anergic, to a specific antigen exhibits substantially reduced or eliminated responses (e.g., proliferation and/or lymphokine production) upon reexposure to the antigen. In one embodiment of the invention, the response of a donor T cell to alloantigens is inhibited to reduce or substantially eliminate graft versus host disease in a bone marrow transplant recipient. In another embodiment, the response of a recipient T cell to alloantigens is inhibited to reduce or substantially eliminate rejection of a donor graft (e.g., transplanted cells, tissue or organ).

To inhibit a T cell response to an antigen according to the methods of the invention, a T cell is contacted with at least one inhibitor of a costimulatory signal in the T cell alone, or in conjunction with another agent. An "inhibitor of a costimulatory signal" or an "agent which inhibits generation of a costimulatory signal" interferes with, blocks or substantially eliminates formation of or delivery of a second signal in the T cell which, together with a first, antigen specific, signal mediated through the TCR/CD3 complex, is necessary to induce an antigen specific response by the T cell. Typically, this second or costimulatory signal is mediated by a T cell surface receptor such as CD28 and/or CTLA4 (or related molecule)

upon interaction with a ligand such as B7-1 and/or B7-2, (or related molecule, e.g., B7-3) on a cell presenting antigen to the T cell (e.g., on a B cell, on a "professional" antigen-presenting cell, or APC, such as a monocyte/macrophage, dendritic cell or Langerhans cell, or another cell type which can present antigen to a T cell, such as a keratinocyte, endothelial cell, astrocyte, fibroblast, or oligodendrocyte). An alternative example of a costimulatory molecule is heat stable-antigen (HSA) (Liu, Y. et al. (1992) *Eur. J. Immunol.* 22, 2855). Ligands such as B7-1, B7-2 or HSA which trigger a costimulatory signal in a T cell through a T cell surface receptor (e.g., CD28) are collectively referred to herein as "costimulatory molecules". T cell surface receptors to which such costimulatory molecules bind (e.g., CD28, CTLA4) are collectively referred to herein as "costimulatory receptors".

Accordingly, in one embodiment of the invention, an inhibitor of a costimulatory signal in a T cell is an agent which inhibits an interaction between a receptor on the T cell and a costimulatory molecule on a cell presenting antigen to the T cell. This type of agent, also referred to herein as a "costimulatory blocking agent" can be a soluble form of the receptor on the T cell (or a related receptor on the T cell which similar binding specificity), a soluble form of the costimulatory molecule(s), or an antibody (or fragment thereof) which binds to either the receptor or the costimulatory molecule. A preferred costimulatory inhibitor is a CTLA4-immunoglobulin fusion protein (CTLA4Ig), a soluble form of the CTLA4 receptor on T cells which binds to both B7-1 and B7-2. In another embodiment, the costimulatory inhibitor acts intracellularly to inhibit generation of or delivery of a costimulatory signal in a T cell by a CD28- and/or CTLA4-associated signal transduction pathway.

In addition to use of an inhibitor of a costimulatory signal to inhibit inappropriate T cell responses to antigen, a second agent which inhibits another T cell function can be used. In one embodiment, the second agent inhibits adhesion of the T cell to a cell presenting antigen to the T cell. Preferably, this second agent inhibits an interaction between an adhesion molecule on a T cell and a ligand for the adhesion molecule on a cell presenting antigen to the T cell (such as the cell types discussed above). The term "adhesion molecule" as used herein refers to a molecule on the surface of a cell whose primary, or predominant, function is to increase the strength or avidity of the interaction of the cell with another cell (e.g., the interaction between a T cell and an APC). Accordingly, a "ligand for an adhesion molecule" can also be considered as an adhesion molecule (i.e., the second agent can inhibit an interaction between two adhesion molecules, one on a T cell and the other on a cell presenting antigen to the T cell). It is possible that an adhesion molecule, or ligand therefor, may serve an additional function(s) (e.g., a signalling function). Examples of families of adhesion molecules include integrins and selectins. In a preferred embodiment, the second agent used to inhibit a T cell response interferes an interaction between the integrin LFA-1 and its ligand(s) ICAM-1, ICAM-2 and/or ICAM-3. Alternatively, the second agent may interfere with the activity of other adhesion molecules such as CD49 a, b, c, d, e and/or f or

equivalents (e.g., VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6) CD29 (fibronectin receptor; integrin beta 1 chain), CD43 (leukosialin), CD48 (an additional LFA-1 ligand), VCAM-1 (a VLA-4 ligand), CD52 (CAMPATH), CD56 (N-CAM), CD59, CD61 (beta chain of VNR; integrin beta 3 chain), CD62P (P-selectin), LECAM-1 (L-selectin or Mel-14), ELAM-1 (E-selectin), CD44 (also called Pgp-1), CD103 (HML-1; integrin aE subunit), CD104 (integrin beta 4 chain), Thy-1 and gp39 (for discussions of adhesion molecules see Janeway, C. et al. (1993) *Curr. Opin. Immunol.* 5:313-323; Mobley, J.L. (1993) *Semin. Immunol.* 5:227-236; Patel, D.D. et al. (1993) *Semin. Immunol.* 5:283-292; Rosen, S.D. et al. (1993) *Semin. Immunol.* 5:237-247; and Picker, L.J. and Butcher, E.C. (1992) *Ann. Rev. Immunol.* 10:561-592). Inhibiting the binding ability of adhesion molecules on endothelial cells may be useful since endothelial cells are targets of GVHD (e.g., lung and liver) and of organ graft rejection (e.g., lung, liver, cardiac and kidney tissues include endothelial cells). The adhesion blocking agent can be, for example, a soluble form of the adhesion molecule or ligand for the adhesion molecule, an antibody (or fragment thereof) which binds either the adhesion molecule or the ligand for the adhesion molecule, or a peptide, peptide mimetic or other form of small soluble molecule (e.g., drug) that inhibits an interaction between an adhesion molecule and a ligand therefor. A preferred second agent is an anti-LFA-1 antibody, or fragment thereof.

In another embodiment of the invention, inhibition of inappropriate T cell responses is accomplished by use of an inhibitor of a costimulatory signal together with a second agent which inhibits generation of or delivery of a proliferative signal in the T cell. An "agent which inhibits generation of a proliferative signal in a T cell" interferes with formation of or delivery of an intracellular signal associated with the interaction of a T cell growth factor with a growth factor receptor on the T cell. Accordingly, in one embodiment, the second agent inhibits an interaction between a receptor on a T cell and T cell growth factor. Preferably, the second agent inhibits an interaction between the T cell growth factor interleukin-2 (IL-2) and an interleukin-2 receptor (IL-2R) on a T cell. Alternatively, the activity of other T cell growth factors and/or their receptors can be targeted for inhibition. Other interleukins involved in stimulation of T cells include interleukin-1α, interleukin-1β, interleukin-2, interleukin-4, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-12, interleukin-15 and interleukin-T. Additionally, interferon α, β and γ, and tumor necrosis factor α and β, have T cell stimulatory capacity. Accordingly, these ctyokines, or receptors therefor, can be targeted for inhibition. In one embodiment, the second agent is an antibody (or fragment thereof) which binds either to a T cell growth factor or to a growth factor receptor on a T cell. A preferred second agent is an anti-IL-2R antibody, or fragment thereof. In another embodiment, the second agent acts intracellularly to inhibit generation of a proliferative signal in a T cell.

Based upon the results observed with the second agents described above, other agents which inhibit other surface molecules involved in T cell interactions and/or T cell activation can be used in conjunction with a costimulation inhibitory agent to inhibit a T cell response.

These and other embodiments of the invention are described in further detail in the following subsections:

I. Agents for Inhibiting a T Cell Response

A. Antibodies

In one embodiment of the invention, an agent used to inhibit an antigen specific T cell response can be an antibody (or fragment thereof). Antibodies suitable for use in the methods of the invention are available in the art (e.g., from the American Type Culture Collection, Rockville, MD, or commercially, e.g., from Becton-Dickinson or Immunotech) or can be prepared by standard techniques for making antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen. Structurally, the simplest naturally occurring antibody (e.g., IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a naturally-occurring antibody. Thus, these antigen-binding fragments are also intended to be designated by the term "antibody". Examples of binding fragments encompassed within the term antibody include (i) an Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) an Fd fragment consisting of the VH and CH1 domains; (iii) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) a dAb fragment (Ward et al., (1989) *Nature* 341:544-546) which consists of a VH domain; (v) an isolated complimentarity determining region (CDR); and (vi) an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described for whole antibodies. The term "antibody" is further intended to include bispecific and chimeric molecules having an antigen binding portion. Furthermore, although the two domains of an Fv fragment are coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird et al. (1988) *Science* 242:423-426; and Huston et al. (1988) *PNAS* 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term "antibody".

To prepare an antibody specific for a molecule to be targeted in the method of the invention (e.g., a costimulatory molecule, an adhesion molecule, a growth factor receptor, etc.), an animal is immunized with an appropriate immunogen. The term "immunogen" is used herein to describe a composition typically containing a protein or peptide as an active

ingredient used for the preparation of antibodies against the protein or peptide. It is to be understood that the protein or peptide can be used alone, or linked to a carrier as a conjugate, or as a peptide polymer. The immunogen should contain an effective, immunogenic amount of the peptide or protein (optionally as a conjugate linked to a carrier). The effective amount of the immunogen per unit dose depends on, among other things, the species of animal inoculated, the body weight of the animal and the chosen immunization regimen, as is well known in the art. The immunogen preparation will typically contain peptide concentrations of about 10 micrograms to about 500 milligrams per immunization dose, preferably about 50 micrograms to about 50 milligrams per dose. An immunization preparation can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

Either soluble or membrane bound protein or peptide fragments are suitable for use as an immunogen. A purified form of protein, such as may be isolated from a natural source or expressed recombinantly by conventional techniques known in the art, can be directly used as an immunogen. Those skilled in the art will appreciate that, instead of using naturally occurring forms of protein for immunization, synthetic peptides can alternatively be employed towards which antibodies can be raised for use in this invention. The purified protein can also be covalently or noncovalently modified with non-proteinaceous materials such as lipids or carbohydrates to enhance immunogenicity or solubility. Alternatively, a purified protein can be coupled with or incorporated into a viral particle, a replicating virus, or other microorganism in order to enhance immunogenicity. It is also possible to immunize an animal with whole cells which express a protein on their surface against which an antibody is to be raised (e.g., T cells or antigen presenting cells expressing surface molecules of interest can be used as immunogens). As yet another alternative, it is possible to use nucleic acid (e.g., DNA) encoding the protein or peptide of interest as an immunogen for so-called genetic immunization. Thus, the term "immunogen" is also intended to include nucleic acid encoding a protein or peptide against which antibodies are to be raised (see e.g., Tang, D.C. et al. (1992) *Nature* 356:152-154; Eisenbraun, M.D. et al. (1993) *DNA Cell Biol.* 12:791-797; Wang, B. et al. (1993) *DNA Cell Biol.* 12:799-805 for descriptions of genetic immunization).

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of an immunogen and an adjuvant. As an illustrative embodiment, animals are typically immunized against a protein, peptide or derivative by combining about 1 μg to 1 mg of protein with Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of immunogen in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for specific antibody titer (e.g., by ELISA). Animals are

boosted until the titer plateaus. Also, aggregating agents such as alum can be used to enhance the immune response.

Such mammalian-produced populations of antibody molecules are referred to as "polyclonal" because the population comprises antibodies with differing immunospecificities and affinities for the immunogen. The antibody molecules are then collected from the mammal (e.g., from the blood) and isolated by well known techniques, such as protein A chromatography, to obtain the IgG fraction. To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid phase-affixed immunogen. The antibody is contacted with the solid phase-affixed immunogen for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. The bound antibodies are separated from the complex by standard techniques.

The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site. A monoclonal antibody composition thus typically displays a single binding affinity for a particular protein with which it immunoreacts. Monoclonal antibodies can be prepared using a technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497; see also Brown et al. (1981) *J. Immunol* 127:539-46; Brown et al. (1980) *J Biol Chem* 255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75) and the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96), and trioma techniques.

Thus, a monoclonal antibody can be produced by the following method, which comprises the steps of:

(a) Immunizing an animal with a protein (or peptide thereof). The immunization is typically accomplished by administering the immunogen to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period sufficient for the mammal to generate high affinity antibody molecules. Antibody production is detected by screening the serum from the mammal with a preparation of the immunogen protein. These screening methods are well known to those of skill in the art, e.g., enzyme-linked immunosorbent assay (ELISA) and/or flow cytometry.

(b) A suspension of antibody-producing cells removed from each immunized mammal secreting the desired antibody is then prepared. After a sufficient time, the animal (e.g., mouse) is sacrificed and somatic antibody-producing lymphocytes are obtained. Antibody-producing cells may be derived from the lymph nodes, spleens and peripheral blood of

primed animals. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiologically tolerable medium using methods well known in the art. Mouse lymphocytes give a higher percentage of stable fusions with the mouse myelomas described below. Rat, rabbit and frog somatic cells can also be used. The spleen cell chromosomes encoding desired immunoglobulins are immortalized by fusing the spleen cells with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of antibody of the desired specificity, e.g., by immunoassay techniques using the antigen that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions and the monoclonal antibody produced can be isolated. Various conventional methods exist for isolation and purification of the monoclonal antibodies so as to free them from other proteins and other contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see, e.g., Zola et al. in Monoclonal Hybridoma Antibodies: Techniques And Applications, Hurell (ed.) pp. 51-52 (CRC Press 1982)). Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art.

Generally, the individual cell line may be propagated *in vitro*, for example in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation. Alternatively, the yield of monoclonal antibody can be enhanced by injecting a sample of the hybridoma into a histocompatible animal of the type used to provide the somatic and myeloma cells for the original fusion. Tumors secreting the specific monoclonal antibody produced by the fused cell hybrid develop in the injected animal. The body fluids of the animal, such as ascites fluid or serum, provide monoclonal antibodies in high concentrations. When human hybridomas or EBV-hybridomas are used, it is necessary to avoid rejection of the xenograft injected into animals such as mice. Immunodeficient or nude mice may be used or the hybridoma may be passaged first into irradiated nude mice as a solid subcutaneous tumor, cultured *in vitro* and then injected intraperitoneally into pristane primed, irradiated nude mice which develop ascites tumors secreting large amounts of specific human monoclonal antibodies.

Media and animals useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al. (1959) *Virol.* 8:396) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal caf serum. An exemplary inbred mouse strain is the Balb/c.

When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Such antibodies are the equivalents of the monoclonal and polyclonal antibodies described above, but may be less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the constant region of a murine (or other species) monoclonal antibody molecule is substituted with a gene encoding a human constant region. (see Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl Cancer Inst.* 80:1553-1559).

A chimeric antibody can be further "humanized" by replacing portions of the variable region not involved in antigen binding with equivalent portions from human variable regions. General reviews of "humanized" chimeric antibodies are provided by Morrison, S. L. (1985) *Science* 229:1202-1207 and by Oi et al. (1986) *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of an immunoglobulin variable region from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from an anti-CTLA4 antibody producing hybridoma. The cDNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (see U.S. Patent 5,225,539 to Winter; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060).

As an alternative to humanizing an mAb from a mouse or other species, a human mAb directed against a human protein can be generated. Transgenic mice carrying human antibody repertoires have been created which can be immunized with human protein or peptide immunogen. Splenocytes from these immunized transgenic mice can then be used to create hybridomas that secrete human mAbs specifically reactive with the human protein (see, e.g., Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT publication WO 92/03918; Kay et al. PCT publication 92/03917; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326; and).

Monoclonal antibodies can also be produced by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry et al. (1989) *PNAS* 86:5728; Huse et al. (1989) *Science* 246:1275; and Orlandi et al. (1989) *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al. (1991) *Methods: Companion to Methods in Enzymology* 2:106-110).

As an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the

primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large diverse antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with a protein, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the protein. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

In another embodiment of the phage display library screening approach, the V region domains of heavy and light chains are expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene is subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with a particular antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

B. Soluble Proteins and Fusion Proteins

In another embodiment of the invention, an agent used to inhibit a T cell response is a soluble form of a molecule on the surface of a T cell (e.g., a costimulatory receptor, growth factor receptor or adhesion molecule) or a molecule on the surface of a cell which presents antigen to the T cell (e.g., a costimulatory molecule or adhesion molecule). This soluble protein is capable of inhibiting an interaction between the surface form of the molecule and its ligand(s) (and/or inhibiting an interaction between a related surface molecule having similar binding specificity and its ligand (s)). For example, soluble forms of CTLA4, B7-1 and or B7-2 can be used. A preferred first agent for use in the described methods is a soluble form of a CTLA4 molecule (in particular, a CTLA4-immunoglobulin fusion protein) which binds to both B7-1 and B7-2, and can inhibit the interaction of B7-1 and B7-2 with CD28 and/or CTLA4.

Soluble forms of surface-bound proteins can be made using standard recombinant DNA and protein expression techniques known in the art. Nucleic acid comprising a nucleotide sequence encoding the extracellular domain (or portion thereof) of a surface-bound protein of interest (i.e., lacking the nucleotide sequence of the transmembrane and cytoplasmic domains) can be isolated and cloned into a standard expression vector, either for expression in prokaryotic or eukaryotic cells. The expression vector is introduced into an appropriate host cell (e.g., *E. coli* for prokaryotic expression; yeast or mammalian cells, e.g., COS, CHO or NS0 cells, for eukaryotic expression) and the cells are cultured to allow for expression of the protein encoded therein. The protein is then purified by standard techniques from harvested host cells or, if the protein is secreted from the cells, from the media in which the cells are cultured.

The extracellular domain (or portion thereof) of a surface-bound protein can be expressed recombinantly as a non-fusion protein, or more preferably, is expressed as a fusion protein with a second protein or polypeptide. As used herein, the term "fusion protein" refers to a protein composed of a first polypeptide operatively linked to a second, heterologous, polypeptide. A preferred type of fusion protein to be used as an agent in the methods of the invention is an immunoglobulin fusion protein (e.g., CTLA4Ig). The term "immunoglobulin fusion protein" refers to a fusion protein in which the second, heterologous polypeptide is an immunglobulin constant region, or portion thereof. Immunoglobulin fusion proteins have been described extensively in the art (see e.g., U.S. Patent No. 5,116,964 by Capon et al.; Capon, D.J. et al. (1989) *Nature* 337:525-531; and Aruffo, A. et al. (1990) *Cell* 61:1303-1313), and typically include at least a functionally active hinge region, CH_2 and CH_3 domains of a constant region of an immunoglobulin heavy chain (e.g., human $C\gamma 1$). Construction of a B7-1-Ig fusion protein and a CD28Ig fusion protein is described in detail in Linsley, P.S. et al. (1991) *J. Exp. Med.* 173:721-730. Construction of a CTLA4Ig fusion protein is described in detail in Linsley, P.S. et al. (1991) *J. Exp. Med.* 174:561-569 and

Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6586. Other Ig fusion proteins (e.g., B7-2-Ig) can be similarly prepared.

C. *Bispecific Agents*

Another aspect of the invention pertains to novel bispecific agents for use in inhibiting inappropriate T cell responses to antigen in clinical situations, such as bone marrow and organ transplantation, as well as autoimmune disorders and allergic responses. Based in part on the discovery that an agent which inhibits of a costimulatory signal in T cells can be used *in vitro* or *in vivo* in conjunction with another agent which either inhibits adhesion of a T cell to a cell presenting antigen to the T cell or inhibits generation of a proliferation signal in T cells, novel bispecific agents or molecules incorporating the functions of both agents can be designed and produced. Accordingly, bispecific agents comprising a first binding specificity for a costimulatory molecule or a costimulatory receptor and a second binding specificity for an adhesion molecule are within the scope of this invention. A first binding specificity for a costimulatory molecule, such as B7-1 or B7-2 can be provided for by an anti-B7-1 antibody, or fragment thereof, or an anti-B7-2 antibody, or fragment thereof. Alternatively, a single antibody which binds both B7-1 and B7-2 can be used. Preferably, the first binding specificity for B7-1 and B7-2 is provided for by an Fv fragment (i.e., an V_H and V_L of an whole antibody). In addition, the B7-1 or B7-2 binding specificity can be provided for by a CTLA4Ig fusion protein. In another embodiment, the first binding specificity is for a costimulatory receptor, such as CD28 or CTLA4. In this embodiment, the CD28 or CTLA4 binding specifiicity is provided for by an anti-CD28 antibody, or fragment thereof (e.g., Fv fragment), or an anti-CTLA4 antibody, or fragment thereof (e.g., Fv fragment).

In addition to a binding specificity for a costimulatory molecule or a costimulatory receptor, the bispecific agents of the invention have a second binding specificity for an adhesion molecule, such as LFA-1 or LFA-1 (as previously described herein) or a growth factor receptor, such as interleukin-2 receptor (IL-2R), or other growth factor receptor (as previously described herein). Thus, the second binding specificity can be provided for by a soluble form of the adhesion molecule/growth factor receptor (e.g., LFA-1Ig fusion/IL-2Ig fusion) or antibody specifically reactive with the adhesion molecule/growth factor receptor or adhesion molecule ligand, or fragment thereof (e.g., Fv fragment).

The novel bispecific agents of the invention can be produced by standard techniques such as those used for the production of bispecific antibodies. For example, bispecific antibodies can be made by fusion of two hybridomas with two different specificities (see e.g., Milstein, C. and Cuello, A.C. (1983) *Nature* 305:537-540). Alternatively, recombinant bispecific fragments can be made, such as by chemical crosslinking of the hinge cysteine residues of two antibodies (see e.g., Shalaby, M.R. et al. (1992) *J. Exp. Med.* 175:217-225) or by including a C-terminal peptide that promotes dimerization (see e.g., Kostelny, S.A. et al.

(1992) *J. Immunol.* 148:1547-1553; and Pack, P. et al. (1992) *Biochemistry* 31:1579-1584). A bispecific agent composed of two linked antibody Fv fragments (i.e., V_H and V_L regions) can be prepared as described in Hollinger, P. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448). Additionally, fusion proteins, such as immunoglobulin fusion proteins (e.g., CTLA4Ig, B7-1-Ig or B7-2-Ig) can be incorporated into a bispecific agent using standard recombinant DNA techniques or chemical crosslinking techniques. For example, the immunoglobulin constant region of the fusion protein can be linked to a second molecule having a second binding specificity (e.g., antibody or fragment thereof).

D. Additional Blocking Agents

Alternative to an antibody (or fragment thereof), soluble receptor or ligand (or portion thereof), or bispecific form of these molecules, other molecules which inhibit interactions between cell surface molecules are within the scope of the invention for use in inhibiting T cell responses. For example, a peptide, peptide mimetic, or other form of small molecule (such as a drug) which inhibits an interaction between a receptor and a costimulatory molecule can be used to inhibit a costimulatory signal in a T cell. Similarly, a peptide, peptide mimetic, or other form of small molecule (such as a drug) which inhibits adhesion or a T cell to a cell presenting antigen to the T cell, or inhibits an interaction between a T cell growth factor and its receptor on a T cell, can be used as a second agent in conjunction with a costimulation inhibitory agent to inhibit a T cell response.

E. Intracellular Agents

In other embodiments of the described methods, an agent which acts intracellularly to interfere with the formation of an intracellular signal(s) associated with a particular signal transduction pathway can be used to inhibit a T cell response. For example, a costimulation inhibitory agent as described herein can be an agent that acts intracellularly to inhibit a CD28- or CTLA4-associated signal transduction pathway. CD28 stimulation has been shown to result in protein tyrosine phosphorylation in T cells (see e.g., Vandenberghe, P. et al. (1992) *J. Exp. Med.* 175:951-960; Lu, Y. et al. (1992) *J. Immunol.* 149:24-29). Accordingly, a tyrosine kinase inhibitor, such as herbimycin A, can be used as a first agent to inhibit a CD28-associated signal transduction pathway, thereby inhibiting generation of a costimulatory signal in the T cell. Alternatively, a CD28-associated signal transduction pathway can be inhibited using an agent which stimulates protein tyrosine phosphatase activity in a T cell, thereby decreasing the net amount of protein tyrosine phosphorylation. For example, an antibody directed against the cellular tyrosine phosphatase CD45 can be used to stimulate tyrosine phosphatase activity in a T cell expressing CD45 on its surface. Other intracellular signals reported to be associated with CD28 ligation include increased phospholipase C activity (see e.g., Nunes, J. et al. (1993) *Biochem. J.* 293:835-842) and increased intracellular calcium levels (see e.g. Ledbetter, J.A. et al. (1990) *Blood* 75:1531-

1539). Accordingly, an agent which inhibits phospholipase C activity and/or inhibits increases in intracellular calcium levels can be used to inhibit the generation of a costimulatory signal in a T cell.

Additionally or alternatively, a second agent, which inhibits generation of a proliferative signal in a T cell (used in conjunction with the costimulation inhibitory agent) can act intracellularly to interfere with formation of an intracellular signal(s) associated with the interaction of a T cell growth factor (e.g., IL-2) with its receptor (e.g., IL-2R). Interleukin-2 has been reported to induce tyrosine phosphorylation in T cells (see e.g., Mills, G. et al. (1990) *J. Biol. Chem.* 265:3561-3567). Accordingly, a tyrosine kinase inhibitor, such as herbimycin A, can be used to inhibit generation of a proliferative signal in a T cell. Additionally, certain immunosuppressive drugs, such as cyclosporin A, function at least in part by inhibiting the production of IL-2 by T cells, thereby interfering with the normal autocrine growth mechanism of T cells. A drug that inhibits the production or function of IL-2, or other T cell growth factor, may thus be useful for inhibiting generation of a proliferative signal in a T cell.

F. Compositions

The first and second agents used according to the described methods to inhibit a T cell response can be formulated into pharmaceutical compositions suitable for administration to a subject *in vivo*. Accordingly, another aspect of the invention pertains to pharmaceutical compositions. A preferred composition of the invention comprises a CTLA4Ig fusion protein and an anti-LFA-1 antibody, in an amount effective to inhibit a T cell response, and a pharmaceutically acceptable carrier. Another preferred composition of the invention comprises a CTLA4Ig fusion protein and an anti-IL-2R antibody, in an amount effective to inhibit a T cell response, and a pharmaceutically acceptable carrier.

The agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to inhibit a T cell response. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the ligand. The term subject is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, and transgenic species thereof.

Administration of a therapeutically active amount of the agents described herein is defined as an amount effective, at dosages and for periods of time necessay to achieve the desired result. For example, a therapeutically active amount of a CTLA4Ig fusion protein, together with a therapeutically active amount of either an anti-LFA-1 antibody or anti-IL-2R antibody, may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of fusion protein and antibody to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response.

For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active agent (e.g., antibody and/or fusion protein) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. To administer an agent by other than parenteral administration, it may be necessary to coat the agent with, or co-administer the agent with, a material to prevent its inactivation. An agent may be administered to an individual in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27). Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active agent in the required amount of an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic

dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., antibody) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

II. Uses of the Invention

The methods of the invention can be used to inhibit T cell responses either *in vitro* or *in vivo* by contacting a T cell with a costimulation inhibitory agent, optionally with a second agent as described herein. Accordingly, the term "contacting" as used herein is intended to include incubating (or culturing) a T cell with the first and second agent and administering a first and second agent to a subject. The methods of the invention are useful in therapeutic situations where it is desirable to inhibit an unwanted T cell response, as described in further detail in the subsections to follow. Additionally, as demonstrated in the Examples, the methods of the invention induce antigenic nonresponsiveness in a T cell that persists after cessation of treatment (i.e., antigenic nonresponsiveness persists in vivo after administration of the first and second agents is stopped). Thus, the methods of the invention are useful for inducing T cell anergy, thereby providing a means for long-term inhibition of T cell responses in a variety of clinical situations without the need for chronic generalized immunosuppression of a subject with its attendant deleterious side effects.

A. Bone Marrow Transplantation - Inhibition of GVHD

The methods of the invention are particularly useful for inhibiting graft versus host disease which results from allogeneic bone marrow transplantation. It has previously been observed that the presence of mature donor T cells within a bone marrow graft is beneficial both for successful engraftment and for a graft versus leukemia response. However, the presence of mature donor T cells in the graft induces GVHD. As demonstrated in the Examples, it is possible to inhibit responses of alloreactive donor T cells by use of a costimulation inhibitory agent (e.g., a costimulation blocking agent, such as CTLA4Ig) or by the combined use of a costimulation inhibitory agent and a second agent which inhibits another donor T cell function. These treatments thus allows mature T cells to be present within transplanted donor cells, thus avoiding GVHD and promoting bone marrow engraftment. Moreover, T cell unresponsiveness to alloantigens is induced, thereby providing long-term inhibition of T cell responses without the need for continuous treatment of the bone marrow recipient.

Because the T cells to be inhibited in a bone marrow transplant situation are donor T cells which are available *in vitro* prior to transplantation, alloreactive donor T cell responses can be be inhibited *in vitro*, *in vivo* or, most preferably, using a combined *in vitro/ in vivo* treatment regiment (see the Examples). Accordingly, in one embodiment, graft versus host disease in a bone marrow transplant recipient is inhibited by contacting a population of donor T cells *in vitro* (prior to transplantation) with 1) a second population of cells expressing recipient alloantigens (such as recipient cells or cells from another source which share recipient alloantigens, e.g., major or minor histocompatibility antigens) and 2) an agent which inhibits a costimulatory signal in a donor T cell. In another embodiment, the donor T cells are contacted with 1) and 2) described above, and 3) a second agent which either inhibits adhesion of a donor T cell to cells expressing recipient alloantigens or inhibits generation of a proliferative signal in the donor T cell. In one embodiment, the agent which inhibits a costimulatory signal (i.e., the first agent) is a CTLA4Ig fusion protein. In another embodiment, the first agent is an anti-B7-1 or anti-B7-2 antibody (or fragment therof) or both anti-B7-1 and anti-B7-2 antibodies (or a single antibody which binds both B7-1 and B7-2). In one embodiment, the second agent is either an anti-LFA-1 antibody or an anti-IL-2R antibody. The second population of cells, which express recipient alloantigens, are typically treated such that they cannot proliferate and/or are not metabolically active, e.g., the cells are irradiated and/or treated with paraformaldehyde.

In the method, the population of donor cells contacted with the inhibitory agent(s) include mature donor T cells. Accordingly, the population of donor cells used in the method can be, for example, the bone marrow cells themselves which are to be transplanted into the recipient which have not been T cell depleted. Alternatively, or additionally, the source of mature donor T cells can be donor peripheral blood cells, splenocytes or other suitable source of donor T cells. When non-bone marrow T cells (e.g., peripheral blood T cells or

splenocytes) are used as the source of mature T cells which are contacted with the inhibitory agent(s), the subsequent bone marrow graft includes a mixture of bone marrow cells and non-bone marrow cells (i.e., bone marrow cells together with mature donor T cells in which alloreactivity has been inhibited).

It has been found that primed T cells are more susceptible to inhibition by the inhibitory agents described herein than unprimed T cells (see Experiment 3 in the Examples). Accordingly, in a preferred embodiment, the *in vitro* treatment regimen involves culturing the donor cells with the recipient cells *in vitro* in the absence of the inhibitory agent(s) prior to adding the inhibitory agent(s) to the culture *in vitro*. For example, donor cells (including donor T cells) are cultured with the second population of cells expressing recipient alloantigens (e.g., recipient hematopoietic cells) in a typical mixed lymphocyte reaction (MLR). The cells are cultured for a suitable length of time to induce alloreactive T cells, e.g. one to three days. This step serves to prime donor alloreactive T cells to recipient alloantigens. Following this priming step, the inhibitory agent(s) are added to the culture, e.g., after about 18 to 36 hours of priming, the inhibitory agents can be added for several hours to the culture prior to transplantation of cells into the recipient.

Following *in vitro* culture of donor cells with cells expressing recipient alloantigens and inhibitory agent(s) (preferably, with priming of donor cells to recipient alloantigens), the donor cells are administered to the recipient (if the donor cells used in the *in vitro* culture do not include bone marrow cells, e.g., if peripheral blood cells or splenocytes are uses as the source of mature donor T cells, then T-cell depleted bone marrow cells are also administered to the recipient).

In another embodiment, following *in vitro* treatment and administration of donor cells to the recipient, the recipient is further treated *in vivo* with the inhibitory agent(s). That is, a costimulation inhibitory agent can be administered to the recipient alone or with a second agent, such as an adhesion blocking agent or a proliferation blocking agent. Alternatively, the second agent alone can be administered to the recipient. In another embodiment, the recipient is only treated *in vivo* with the inhibitory agent(s) (i.e., by administering the agent(s) to the recipient). In this embodiment, the *in vitro* culture of donor and recipient cells, and treatment thereof with one or more inhibitory agents, is omitted.

B. Tissue and Organ Transplantation

The methods of the invention can also be applied to other transplant situations, such as transplantation of allogeneic cells, such as allogeneic cells present within a tissue or organ (e.g., pancreatic islets, skin, heart, liver, lung, kidney etc.), to inhibit rejection of the allogeneic cells by the recipient. To inhibit rejection of allogeneic cells in a transplant recipient, a combination of two agents is administered to the recipient: 1) a first agent which inhibits a costimulatory signal in a recipient T cell, and 2) a second agent which either inhibits adhesion of a recipient T cell to a cell that presents antigen to the recipient T cell or

inhibits a proliferative signal in the recipient T cell. For example, the first agent can be a CTLA4Ig fusion protein, anti-B7-1 antibody or anti-B7-2 antibody (or antibody which binds both B7-1 and B7-2). The second agent can be, for example, either an anti-LFA-1 antibody or an anti-IL-2R antibody.

Since, as previously discussed above, primed alloreactive cells may be more susceptible to inhibition by the combined first and second agents than unprimed T cells, the method for inhibiting rejection of a graft in a transplant recipient can include a pretreatment step involving administration of donor cells (e.g., donor hematopoietic cells) to the recipient prior to transplantation of a tissue or organ graft to prime recipient T cells to donor alloantigens. After this pretreatment, the tissue or organ graft is transplanted and the first and second inhibitory agents are administered to the recipient. The responses of primed donor-specific alloreactive T cells in the recipient are inhibited upon subsequent exposure to donor alloantigens within the graft in the presence of the first and second inhibitory agents.

Additonally or alternatively, the method for inhibiting rejection of allogeneic cells by a recipient can involve pretreatment of the graft *ex vivo* with the inhibitory agents described herein prior to transplantation into the recipient. For example, antibodies, fusion proteins or other inhibitory agents can be incubated with allogeneic cells or tissues, or perfused into organs (e.g., the inhibitory agents can be introduced into the organ, the input and output vessels can be clamped to allow the agents to bind to target molecules within the organ, and then the organ can be transplanted into the recipient). Following transplantation, the recipient can be further treated with the inhibitory agents *in vivo* (e.g., by administering the agents to the recipient).

C. Autoimmunity

Inhibition of T cell responses according to the methods of the invention may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue (i.e., reactive against autoantigens) and promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells thus may reduce or eliminate disease symptoms. Administration to a subject suffering from an autoimmune disease of a costimulation inhibitory agent in conjunction with a second agent for inhibiting T cell responses as described herein can be used to prevent the production of autoantibodies or T cell-derived cytokines which may be involved in the disease process. To treat an autoimmune disorder, a first agent (e.g., CTLA4Ig) and a second agent (e.g., anti-LFA-1 mAb or anti-IL2R mAb) are coadministered to a subject in need of treatment. Alternatively, for autoimmune disorders with a known autoantigen, the autoantigen can be coadministered to the subject with first and second agents.

This method may be useful in the treatment of a variety of autoimmune diseases and disorders having an autoimmune component, including diabetes mellitus, arthritis (including

rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

D. Allergy

The IgE antibody response in atopic allergy is highly T cell dependent and, thus, inhibiting responses by allergen-specific T cells may be useful therapeutically in the treatment of allergy and allergic reactions. For example, a combination of a costimulation inhibitory agent and second agent as described herein can be administered to an allergic subject exposed to an allergen to inhibit responses by allergen-specific T cells, thereby downmodulating allergic responses in the subject. Administration of the first and second agents may be accompanied by enviromental exposure to the allergen or by coadministration of the allergen to the subject. Allergic reactions may be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, it may be necessary to inhibit allergen-specific T cell responses locally or systemically by proper administration of the first and second agents. For example, in one embodiment, a first agent (e.g., CTLA4Ig), a second agent (e.g., anti-LFA-1 mAb or anti-IL-2R mAb) and an allergen are coadministered subcutaneously to an allergic subject.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

In the examples, an animal model for graft versus host disease was used to examine the ability of various agents to inhibit T cell responses, as assessed by inhibition of GVHD. In this system, C57BL/6 (B6)(H-2^k) donor cells are transplanted into B10.BR/SgSnJ (B10.BR)(H-2^b) recipient mice. Thus, this system utilizes donors and recipients that are fully mismatched at histocompatibility loci. Efficacy of a therapy in this animal model may be predictive of efficacy in humans, both in unrelated donor and matched sibling donor BMT. This system has previously been used to evaluate other therapies for inhibiting GVHD, such as treatment with rapamycin (Blazar, B.R. et al. (1993) *J. Immunol.* 151:5726-5741; Blazar, B.R. et al. (1993) *Ann. N.Y. Acad. Sci.* 685:73-85), ricin immunotoxins (Vallera, D.A. et al. (1991) *Blood* 77:182-194), radiolabeled anti-Ly-1 (Vallera, D.A. et al. (1991) *Cancer Research* 51:1891-1897) and anti-CD3 (Blazar, B.R. et al. (1993) *J. Immunol.* 150:265-277; Blazar, B.R. et al. (1994) *J. Immunol.* 152:3665-3674), .

In the system, the donor cells comprise a mixture of T cell-depleted bone marrow and splenocytes, as a source of mature T lymphocytes. Agents used to inhibit T cell responses, either alone or in combination, were a CTLA4Ig fusion protein, an anti-LFA-1 antibody and an anti-IL-2 receptor antibody. Different treatment regimens were also examined. Some recipient animals were treated with the various agents only *in vivo*, starting the day before bone marrow transplantation. For other recipient animals, a combined *in vitro/in vivo* treatment regimen was used. In this regimen, prior to transplantation, the donor splenocytes were incubated *in vitro* with irradiated recipient cells, in the presence or absence of the various inhibitory agents (e.g., antibodies and/or fusion protein). In some experiments, donor splenocytes were first cultured with irradiated recipient cells in a mixed lymphocyte reaction (MLR) in the absence of any inhibitory agents (to prime alloreactive donor T cells), followed by addition of the various inhibitory agents to the MLR culture for several hours prior to transplantation of the donor cells. Treatment of the recipient animals was then continued by *in vivo* administration of the various inhibitory agents. In yet another treatment regimen, donor splenocytes were cultured *in vitro* with irradiated recipient cells in the presence of CTLA4Ig or with paraformaldehyde-fixed recipient cells alone, followed by transplantation without further *in vivo* treatment of the recipients. The severity of graft versus host disease was determined by a number of assays, including survival time of the recipient mice in days post-BMT and mean body weight of the recipient mice post-BMT (body weight decreases as a side effect of GVHD).

The following methodology was used in the examples:

Test Animals

B10.BR/SgSnJ (H-2^k) recipient mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2^b) donor mice were purchased form the National Institutes of

Health (Bethesda, MD). Donors and recipients were female. Female donors were 4-6 weeks and female recipients were 8-10 weeks at the time of BMT.

Bone marrow transplantation

The transplant protocol used herein has been described in detail (Blazar, B.R. et al. (1990) *Blood* 75:798). B10.BR recipients were conditioned with 8.0 Gray (Gy) total body irradiation (TBI) administered from a Philips RT 250 Orthovoltage Therapy Unit (Philips Medical Systems, Germany) filtered through 0.35 mm Cu at a final absorbed dose rate of 0.41 Gy/minute at 225 kV and 17 mA. Donor bone marrow (BM) was collected in RPMI 1640 medium by flushing it from the shafts of femurs and tibias. Recipients (8 mice per group per experiment) received 25 x 10^6 BM cells from C57BL/6 donors that had been T-cell depleted (TCD) with anti-Thy1.2 antibody (mAb) (hybridoma 30-H-12, rat IgG2b, provided by Dr. David Sachs, Cambridge, MA) + C' as previously described (see Blazar, B.R.et al. (1990) *Blood* 75:798; Blazar, B.R. et al. (1991) *Blood* 78:3093; Blazar, B.R. et al. (1991) *J. Immunol.* 147:1492) mixed with splenocytes as a source of GVHD-causing T lymphocytes. Single cell suspensions of splenocytes were obtained by passing minced spleens through a wire mesh and collecting them into RPMI 1640. Splenocytes were suspended with BM at a concentration of 10^8 cells/ml to create a bone marrow-splenocyte population (BMS). A 0.5 ml aliquot of BMS, containing 25 x 10^6 BM + 25 x 10^6 splenocytes (50 x 10^6 cells total), was infused into each recipient mouse via caudal vein injection. As a positive control for GVHD prevention in all experiments, one experimental group received splenocytes in which T cells were eliminated with anti-Thy1.2 + C'. This treatment has been previously observed to eliminate all detectable natural killer cell function (Blazar, B.R. et al. (1988) *Transplantation* 45:876) and at least 95 % of cytolytic T lymphocyte precursers (Blazar, B.R. et al. (1990) *Blood* 75:798).

hCTLA4-Ig protein preparation

hCTLA4-Ig (constructed as described in Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6586) was purified by reacting with immobilized protein A supernatants from protein A Chinese hamster ovary cells, electroporated with an expression vector containing the extracellular portion of hCTLA4 joined to the CH_1-Hinge-CH_2-CH_3 domains derived from a human genomic IgG1 gene, with immobilized protein A. Purified hCTLA4-Ig consisted entirely of the dimeric form.

Anti-LFA-1 mAb preparation

An anti-LFA-1 alpha chain mAb (anti-CD11α) (hybridoma FD441.8, rat IgG2b, kindly provided by Dr. Frank Fitch, University of Chicago, Chicago, IL; described in Sarmiento, M. et al. (1982) *Immunol. Rev.* 68:135) was used in the experiments. Ascites fluid from pristane primed mice was concentrated by ammonium sulfate (50 %) precipitation

and was dialyzed against 0.1 M potassium phosphate (pH 7.5) (as described in Blazar, B.R. et al. (1991) *Blood* 78:3093-3102).

Anti-IL-2R mAb preparation

An anti-IL-2R mAb (hybridoma PC615.3, rat IgG1, obtained from the American Type Culture Collection, Rockville, MD) was used in the experiments. Ascites fluid from pristane primed mice was concentrated by ammonium sulfate (50 %) precipitation and was dialyzed against 0.1 M potassium phosphate (pH 7.5) (as described in Blazar, B.R. et al. (1991) *Blood* 78:3093-3102).

In vivo administration of agents

For *in vivo* administration, mice were injected intraperitoneally (ip) with PBS, hCTLA4-Ig (250 μg/dose on days -1, 0 and then 100 μg/dose thrice weekly until day 28 post-BMT), and/or either anti-LFA-1 mAb (300 μg/dose beginning on day -1 and continuing twice weekly through day 29 post-BMT) or anti-IL-2R mAb (250 μg/dose beginning on day -1 through day +5, then 100 μg on days +6 to +10, then continuing 100 μg thrice weekly through day 29 post-BMT).

In vitro incubation of splenocytes with agents

To reduce the capacity of donor GVHD-causing splenocytes to respond to host alloantigens, an *in vitro* culture system was established in which donor splenocytes were exposed to host splenic alloantigens under conditions favoring the development of donor anti-host specific hyporesponsiveness. In initial experiments, donor C57BL/6 splenocytes were suspended at a concentration of 8×10^6/ml in Dulbecco's minimal essential medium (DMEM), 10 % fetal calf sera (commercial brand Hyclone), 2-mercaptoethanol (x 10-M^{-1}), and amino acid and antibiotic supplements. Irradiated (30-33 Gy) host splenocytes, suspended in the media, sera, and supplements described above, were sham treated or incubated with hCTLA4-Ig (50 μg/ml) and/or various mAbs (150 μg/ml) for 30 minutes at 4°C. At the end of this brief incubation, the suspension was not washed, but was diluted to a final concentration of 8×10^6 cells/ml and then combined with the donor splenocytes. Up to 9.6×10^9 each of donor and host splenocytes were added in a total of 150 ml in 225 cm^2 flasks and placed at 37 °C and 10 % $C0_2$ for a period of 2.5 to 4 days as indicated.

In other experiments, the *in vitro* treatment protocol was altered to target donor anti-host primed cells. The *in vitro* culture system described above was modified to first allow a 3 day time period for priming donor splenocytes to irradiated host alloantigens. At the end of the incubation period, the bulk culture was washed, resuspended at a concentration of 50 x 10^6/ml and combined with 50 x 10^6/ml anti-Thy1.2 + C' treated BM to create a BMS preparation. hCTLA4-Ig (50 μg/ml final concentration) and/or anti-LFA1 mAb (150 μg/ml final concentration) or PBS were added to the BMS preparation for 3 hours at 37°C to permit

B7 ligand saturation. The BMS preparation was infused without additional manipulation. Each recipient received 0.5 ml containing 25 x 10^6 BM cells and 25 x 10^6 splenocytes via caudal vein injection. As indicated, recipient groups received PBS, hCTLA4-Ig, and/or anti-LFA1 *in vivo* according to the dose and schedule detailed above and as indicated in the Results sections below.

In some experiments, an alternative approach to reduce T cell costimulation was compared to hCTLA4Ig incubation. Rather than irradiate stimulators, B10.BR stimulator splenocytes (20 x 10^6/ml) were incubated with paraformaldehyde (0.15%) for 60 minutes at 4 °C. Stimulator cells were then washed, resuspended at 8 x 10^6/ml and combined with 8 x 10^6/ml responder cells (B6 splenocytes) for a period of 2.5 or 4 days as a priming step. The responder cells were then combined with donor BM cells, to form a BMS preparation, and the BMS preparation was administered to recipient animals as described above.

In representative experiments, aliquots of the *in vitro* incubated bulk cell population were removed for flow cytometry (FCM) and/or assessment of proliferation by tritiated thymidine incorporation by conventional procedures. In all experiments, at the end of the 2.5-4 day incubation period, the bulk population was washed three time with media, resuspended, and added at a 1:1 ratio to anti-Thy1.2+C' treated BM.

Statistical Analyses

Group comparison of continuous data were made by Student's t-test. Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of chi-square (Mantel, N. (1966) *Cancer Chemother. Rep.* 50:163). Probability (p) values <0.05 were considered significant.

Experiment 1

In this experiment, the GVHD-inhibitory effect of using hCTLA4Ig alone or together with anti-LFA-1 or anti-IL-2R was evaluated in a combined *in vitro/in vivo* treatment regimen (as described above in the Methodology). The following treatment conditions were used:

In Vitro Treatment

hCTLA4-Ig 50 µg/ml added at the end of a 3 day MLR of donor splenocytes and irradiated recipient cells, for 3 hours at 37 °C, either alone or in combination with either anti-IL2-R or anti-LFA-1, each at 150 µg/ml.

In Vivo Treatment

hCTLA4Ig 250 μg on days -1 and 0, then 100 μg thrice weekly through day 28, alone or with either:

anti-IL-2R 250 μg on days -1 to +5, then 100 μg on days 6-10, then thrice weekly through 28

anti-LFA-1 300 μg on days -1 through +29 twice weekly.

The results are shown in Figure 1, which depicts the proportion of surviving mice as a function of the number of days post-BMT, and Figure 2, which depicts the mean weight in grams of the mice as a function of days post-BMT. The 50 % survival rate of control (PBS treated) animals was only about 25 days post-BMT, whereas 50 % of mice treated with CTLA4Ig survived about 60 days post-BMT. Greater than 50 % of mice treated with either CTLA4Ig and anti-LFA-1, or CTLA4Ig and anti-IL-2R, survived beyond 100 days post-BMT. Since all *in vivo* treatment was stopped by day 29 post-BMT, the results indicate that CTLA4Ig treatment *in vitro* and *in vivo*, alone or in combination with anti-LFA-1 or anti-IL-2R, can induce long-term inhibition of T cell responses, consistent with induction of T cell unresponsiveness, or anergy, to alloantigens.

Experiment 2

In this experiment, the following treatment regimens were evaluated:

CTLA4Ig alone, *in vitro* and *in vivo*
anti-LFA-1 alone, *in vitro* and *in vivo*
CTLA4Ig + anti-LFA-1, *in vivo* only
CTLA4Ig + anti-LFA-1, *in vitro* and *in vivo*

The conditions for the treatments were the same as described in Experiment 1. The results are shown in Figure 3 (proportion surviving) and Figure 4 (mean weight in grams). CTLA4Ig or anti-LFA-1 treatment alone (*in vitro* and *in vivo*) prolonged the 50 % survival rate of the animals compared to PBS treated control animals. Coadministration of CTLA4Ig and anti-LFA-1 (*in vivo* only) prolonged the survival rate even longer compared to control treated animals. Animals treated both *in vitro* and *in vivo* with a combination of CTLA4Ig and anti-LFA-1 exhibited the longest survival rate post-BMT compared to control animals.

Experiment 3

In this experiment, a combined *in vitro/in vivo* treatment regimen was used with CTLA4Ig together with anti-LFA-1. Donor splenocytes were cultured with irradiated recipient cells for 3 days in an MLR prior to addition of CTLA4Ig and anti-LFA-1 for the final three hours of culture ("primed" cells). Alternatively, donor splenocytes were cultured with irradiated recipient cells in the continuous presence of CTLA4Ig and anti-LFA-1 ("non-primed"). Treatment of the recipient mice with CTLA4Ig and anti-LFA-1 was continued *in vivo* following transplantation. The treatment conditions were as described in Experiment 1. The results are shown graphically in Figure 5 (proportion surviving) and Figure 6 (mean weight in grams). Recipients of non-primed splenocytes cultured *in vitro* with CLTA4Ig and anti-LFA-1 exhibited longer survival post-BMT compared to control (PBS) treated mice. Preculture of donor splenocytes with recipient cells (i.e., priming) prior to culture with CTLA4Ig and anti-LFA-1 *in vitro* resulted in even longer survival post-BMT compared to control treated animals. The results indicate that while priming of donor T cells to recipient alloantigens prior to treatment is not essential for the GVHD inhibitory effects of CTLA4Ig and anti-LFA-1, inclusion of the priming step in the treatment regimen can increase the inhibitory effects of the agents. This data is consistent with primed T cells being more susceptible to inhibition (e.g., anergy induction) than non-primed T cells.

Experiment 4

In this experiment, the following treatment regimens were evaluated:

CTLA4Ig alone, *in vitro* and *in vivo*
CTLA4Ig + anti-IL-2R, *in vitro*, then CTLA4Ig alone *in vivo*
CTLA4Ig + anti-IL-2R, *in vitro* and *in vivo*
CTLA4Ig + anti-LFA-1, *in vitro* and *in vivo*

The conditions for the treatments were the same as described in Experiment 1. The results are shown in Figure 7 (proportion surviving) and Figure 8 (mean weight in grams). The use of CTLA4Ig alone (*in vitro* and *in vivo*) extended the 50 % survival rate of recipients compared to control (PBS) treated animals (about 60 days vs. about 42 days, respectively). The combined use of CTLA4Ig and anti-LFA-1, or CTLA4Ig and anti-IL2R, prolonged the survival rate of the animals even further (50 % survival greater than 100 days for either treatment). Again, inhibition of GVHD was long-term and persisted after cessation of *in vivo* treatment, consistent with induction of T cell unresponsiveness, or anergy, to alloantigens.

Experiment 5

In this experiment, *in vitro* anergy induction by CTLA4Ig alone was examined. B6 splenocytes (8 x 10^6) were incubated with irradiated B10.BR stimulators (1:1 ratio) in the presence and absence of hCTLA4Ig (50 μg/ml). A third group of donor splenocytes was incubated with non-irradiated, paraformaldehyde-fixed (0.15 %) host splenocytes. Paraformaldehyde fixation prevents the release of and/or induction and surface expression of costimulatory molecules on host APCs. Splenocytes were maintained for 3 or 4 days before overnight pulsing with tritiated thymidine. Δ cpm ($cpm_{experimental}$ - $cpm_{autologous\ response}$) are listed as mean values x 10^{-3}. The % recovery is # responders remaining on the day listed divided by input cell # x 100 %.

	day 3		day 4	
In vitro treatment	% recovery	Δ cpm (% control)	% recovery	Δ cpm (% control)
control	63	33.1 (100 %)	83	25.4 (100 %)
hCTLA4Ig (50 μg/ml)	56	6.1 (18 %)	45	11.7 (46 %)
paraformaldehyde	35	4.2 (13 %)	35	10.6 (42 %)

A 3 day culture is preferable to a 4 day culture, since the 3 day culture had a higher % recovery and a lower % of the control donor anti-host proliferative response in the treated groups. The data demonstrate that the responder cells have been induced into a state of hyporesponsiveness, since (even after adjusting for the difference in % recovery with the control group) the treated groups, as compared to the control group, havea reduced proliferative response to the host. Anti-host responses in the treated groups increased on day 4, suggesting that the frequency of donor anti-host responsive cells was lowered and a longer time was required for expansion as compared to controls.

To determine if this degree of inhibition was sufficient for GVHD protection *in vivo*, a 3 day MLR culture of the three groups was set up. At the end of the 3 days, the cells were extensively washed, resuspended and reinfused (25 x 10^6 cells total) along with 25 x 10^6 T cell-depleted bone marrow cells into B10.BR recipients (n=8 mice/group). An aliquot of the donor cells was plated and pulsed with tritiated thymidine for 18 hours. Mean cpm ± 1 standard error of the mean (SEM) x 10^{-3} = 0.9 ± 0.1 for B6 autologous responses. Autologous cpms have been subtracted from the total cpm to generate the % control response.

In vitro treatment	mean cpm (± 1 SEM)	% control response
allogeneic control	40.4 (0.7)	100
hCTLA4Ig	13.1 (0.5)	31
0.15 % paraformaldehyde	7.7 (0.6)	17

The actuarial survival plot for the recipient mice is shown in Figure 9. The results indicate that donor anti-host responses are reproducible for CTLA4Ig and paraformaldehyde-fixation groups. Despite the fact that an identical number of 100 % viable cells were infused, recipients of either CTLA4Ig added to irradiated host APCs or paraformaldehyde-fixed APCs had higher actuarial survival rates as compared to the control incubated group ($p= 0.056$, 0.057, respectively). The GVHD protective effect associated with a 69-83 % inhibition of donor anti-host responsiveness may have been minimized because the number of cultured splenocytes was so large (5×10^6 fresh splenocytes is an LD_{95} for this strain combination). However, the results clearly demonstrate that *in vitro* donor cell functional manipulation without any T cell depletion can reduce GVHD. It is important to emphasize that no *in vivo* agents were given, the T cell constituency was unchanged in the treated as compared to the control incubated group, and an identical number of viable cells were given. The GVHD protective effect was entirely the result of functional donor T cell alterations prior to BMT.

Experiment 6

In this experiment, phenotypic and functional studies were performed on B6 splenocytes cultured *in vitro* with allogeneic cells.

Phenotypic Analysis

To determine which cells were preferentially expanding *in vitro* in the C57BL/6 anti-B10.BR system and what activation antigens were being induced, 1° mixed lymphocyte reaction (MLR) cells were washed, harvested and phenotyped. Listed are the % positive cells.

Group	CD3e	B7-1 CD3e	IL-2R CD3e	CD69 CD3e	CD28 CD3e	CD3e CD4	CD45RB CD4	CD3e CD8	B220	B7-1 B220	IL-2R B220
no culture	39	3	3	2	39	20	7	14	58	3	3
3 day MLR	58	9	12	12	58	21	14	16	57	15	14

Primed responders had increased proportions of activated T cells, indicated by an increase in the percentage of $CD3e^+$ cells that expressed the activation antigens IL-2Rα chain, B7-1, and CD69. B-cells were also activated and expressed B7-1 and IL-2R (indicated by an increase in the percentage of $B220^+$ cells expressing B7-1 and IL-2R). $CD4^+$ T cells were induced to express CD45RB, an antigen found on IL-2 secreting Th "memory" cells.

The proportion of $CD4^+$ and $CD8^+$ T cells did not change, in contrast to the disproportionate increase in alloactivated $CD8^+$ vs. $CD4^+$ T cells in mice undergoing GVHD *in vivo*.

Functional Analysis

In functional studies, the induction of B6 anti-bm12-specific hyporesponsiveness was evaluated in primary (1°) and secondary (2°) MLR cultures.

Optimized 1° MLR culture conditions were determined to include: 1) T cell depletion of stimulator cells, which reduced background responses; 2) responder:stimulator ratios of 1:1, 1:2, and 1:3 (8×10^6 B6 responders/ml + 8-24 x 10^6 irradiated bm12 stimulators/ml) set-up in bulk culture [These conditions provide roughly equivalent proliferative responses, as measured by Δcpm determinations in a tritriated thymidine incorporation assay. The assay was performed by removing an aliquot at the end of the culture period, placing 1×10^5 or 3×10^5 cells into 96-well plates, pulsing overnight and then harvesting. The proliferative response peaked on day 5 of culture (average cpm range from 28.3-37.2 x 10^3 for 3×10^5 cells plated and 10.9 - 22.5 x 10^3 for 1×10^5 cells plated) and declines on days 6 and 7]; and 3) precursor frequencies of B6 anti-bm12 cells proliferating in a 7 day limiting dilution assay (LDA) ranging from 1:1072 to 1:3696.

To optimize the conditions for 2° MLR cultures, several bulk MLR culture experiments were performed with B6 anti-bm12. Using a 1:1 responder:stimulator ratio (8×10^6 cells/ml each added to 225 cm^2 flasks), the days of 1° MLR were varied (4-6 days), Ficoll-Hypaque density gradient centrifugation was performed after 1° culture to remove residual stimulators and non-viable cells and to enrich for alloreactive populations (blast cells). Also, the number of days of rest between 1° and 2° MLR cultures was varied (1-3 days). These manipulations did not result in a decrease in high autologous responses.

As a measurement of anergy induction in manipulated 1° MLR cultured cells, a limiting dilution assay (LDA) analysis of B6 anti-bm12 proliferating T cell frequencies was performed. Two groups were compared for 1° and 2° MLR cultures. These groups differed only in the presence or absence of hCTLA4-Ig (50 μg/ml) added at the time of 1° MLR culture initiation. Bulk cultures of responders (B6 splenocytes) and irradiated, T cell depleted stimulators (B10.BR splenocytes) (1:1 responder: stimulator ratio; 5×10^6/ml each in 225 cm^2 flasks) were established according to conditions described in the Methodology section above. Following 4 days of incubation, an aliquot of cells was removed from each flask, placed into a 96-well plate, and pulsed overnight with tritiated thymidine to measure proliferation (1° MLR culture results).

The remaining cells were cultured for 5 days, placed on a Ficoll-Hypaque gradient, washed 2 times, and counted to determine the overall yield after processing the 1° MLR cultured cells. These cells were then rested 2 days. Following the 2 days of rest (determined to be the optimal rest period), cells were washed and plated in limiting dilution (5×10^4 -

1.5 x 10^2 at 3-fold dilutions) with irradiated bm12 stimulators (5 x 10^5/well) or irradiated third party (B10.BR: H-2^k) (5 x 10^5/well) stimulators. Wells were supplemented with 10 units/ml rhIL-2 (Hoffman-LaRoche). After 7 days of incubation, cells were pulsed with tritiated thymidine to measure proliferation (2° MLR culture results).

The results of the 1° MLR and 2° MLR (LDA analysis) are shown below:

	--------1° MLR-----		----------2°MLR--------	
Group	Δcpm[1] x 10^{-3} (% reduction)[1]	% recovery[2]	anti-bm12[3] (% reduction)[1]	anti-B10.BR[3] (% reduction)[1]
B6 anti-bm12	12.6	42%	1/341	1/335
B6 anti-bm12 + hCTLA4-Ig	0.3 (98%)	18%	1/1231 (72%)	1/282 (0%)

[1]Δcpm=$cpm_{experimental}$ - $cpm_{background}$. Background responses were determined from cpm results using B6 responders alone. The % reduction is calculated as compared to the B6 anti-bm12control group.

[2]% Recovery is calculated after 5 day MLR, Ficoll-Hypaque centrifugation and 2 washes. Cells had high viability in both groups.

[3]Data are expressed as frequency of responding cells against the indicated stimulators. Probability calculations indicated that the response fit a Poison (single-hit) distribution.

From this experiment, it can be concluded that the B6 anti-bm12 alloresponse is highly susceptible to hCTLA4-Ig-mediated inhibition. The secondary MLR responses can be more readily quantified using LDA than re-establishing bulk cultures. A high degree of alloantigen-specific non-responsiveness in naive splenocytes has been achieved in the 1° MLR bulk cultures as confirmed by 2° MLR responses against the original and a third party stimulator. These results provide evidence that alloantigen-specific hyporesponsiveness can be obtained in fresh splenocytes by *in vitro* CTLA4Ig treatment.

Experiment 7

In this experiment, a phenotypic analysis of GVHD target organs was performed to evaluate the kinetics and type of tissue inflammatory response in BMT recipients of fully allogeneic BMS that have active GVHD. Samples of spleen and GVHD target organs (liver, colon, lung, and skin) were obtained from lethally irradiated B10.BR recipients of C5BL/6 T cell depleted bone marrow plus supplemental splenocytes (25 x 10^6 each) (as described in the Methodology section above). Samples were obtained daily beginning on day + 2 post-BMT and subjected to immunoperoxidase (avidin-biotin-peroxidase) staining with antibodies directed against CD3e, CD4, CD8, Mac-1, MHC class II, B7-1, B7-2, ICAM-1 and ICAM-2.

Lung and colon stained frozen sections are summarized from tissues taken 5 days post-BMT. The major initial cellular influx in the lung and colon peaked on day 5 and in the liver, on day 9. Controls contained only sparse cells positive for T cell or B7 antigens in the colon. A summary of the results is listed below according to the relative proportion of cells expressing each surface antigen: high, moderate (mod), low or none (-). Recipients of TCD BMS have virtually no detectable cells expressing CD3e, CD4, CD8, B7-1, or B7-2 and low numbers of Mac-1^+ and MHC class II^+ cells.

Tissue	CD3e	CD4	CD8	Mac-1	MHC II	B7-1	B7-2	ICAM1	ICAM2
lung	high	low	high	high	high	high	low		
colon	high	low	mod	low	low	-	high	mod	high
liver	mod	low	low	high	low	-	mod		
spleen								high	low

From these studies, it can be concluded that $CD8^+$ T cells are the predominant inflammatory cells present in the lung and colon early post-BMT. Also, B7-2 is induced to a high level in colon and liver by day 5 post-BMT. At this time, while B7-2 is also upregulated in the lung, the predominant B7 antigen is B7-1. The upregulation of B7 molecules could serve as a costimulatory ligand for T cell activation and expansion. The co-expression of high densities of MHC class II antigens in the lung (presumably on alveolar macrophages) could further amplify the activation/expansion process. ICAM-1 and ICAM-2 are upregulated in the spleen of mice undergoing GVHD and, for ICAM-2, in the colon as well.

Experiment 8

In this experiment, the effect of anti-B7-1 and/or anti-B7-2 antibody treatment on the development of GVHD was analyzed.

For this experiment, 10^5 CD4+ T cells obtained from lymph nodes of B6 Ly5.2 mice were injected into irradiated (600 Cs) bm12 recipient mice (day 0). The transplanted mice were further left untreated (PBS) or treated *in vivo* with either of the following regimens:

hCTLA4Ig at 250 μg at day -1 and 0 and then 100 μg three times a week;

anti-B7-1 antibody at 250 μg at day -1 and 0 and then 100 μg three times a week;

anti-B7-2 antibody at 250 μg at day -1 and 0 and then 100 μg three times a week; and

a combination of anti-B7-1 and anti-B7-2 antibodies at 250 μg at day -1 and 0 and then 100 μg three times a week.

Anti-B7-1 and anti-B7-2 monoclonal antibodies have been described, e.g., Freedman, A.S. et al. (1987) *J. Immunol.* 139, 3260 (anti-B7-1), Chen, C. et al. (1994) *J. Immunol.* 152, 2105 and Hathcock, K.S. et al (1993) *Science* 262, 905 (anti-B7-2).

The percent survival of the mice having received the different regimens is represented in Figure 10. The results indicate that administration of either anti-B7-1 or anti-B7-2 antibodies alone prolonged survival of the mice. Moreover, administration of both anti-B7-1 and anti-B7-2 antibodies together to the transplant recipient mice completely protected the mice from GVHD (i.e., 100% survival of the animals was observed).

Thus, administration of either anti-B7-1 or anti-B7-2 antibodies alone significantly inhibits development of GVHD and, more preferably, administration of a combination of both antibodies leads to long term protection from GVHD.

Experiment 9

In this example, the effect of anti-IL-12 or anti-IFN-γ antibody treatment on the development of GVHD was investigated.

In this example, 10^5 CD4+ T cells obtained from lymph nodes of B6 Ly5.2 mice were injected into irradiated (600 Cs) bm12 recipient mice (day 0). The transplanted mice were either left untreated (PBS) or were treated *in vivo* with one of the following regimens:

anti-IL12 (C15.1 and C17.15) at 300 μg twice a week from day -1 to day 21;
anti-IFN-γ (R4-6A2) at 300 μg twice a week from day -1 to day 21; and
anti-B7-1 (1G10.F9) and anti-B7-2 (GL-1) at 250 μg on day -1 and 0 and then at 100 μg three times a week until day 21.

The percent survival of the mice transplanted and treated according to the above described protocol is shown in Figure 11. The results indicate that 100% of the mice having received the anti-IFN-γ antibody survived until at least day 26 following the injection of the alloreactive CD4+ T cells. Administration of a combination of an anti-B7-1 and an anti-B7-2 antibody to the mice also resulted in 100% survival of the mice (confirming the results of Example 8). Administration of anti-IL-12 antibodies was somewhat less efficient than the two previous treatments, but still resulted in protection of approximately 25% of the mice against GVHD.

Experiment 10

In the following example, the effect of administration of anti-CD2 in combination with anti-CD48, or anti-gp39 antibodies alone was examined.

10^6 CD4+ T cells obtained from lymph nodes of B6 Ly5.2 mice were injected into irradiated (600 Cs) bm12 recipient mice (day 0). The transplanted mice were either left untreated (PBS) or were treated with one of the following regimens:

anti-CD2 and anti-CD48 antibodies at 300 μg two times a week starting at day -1;.
anti-gp39 antibody at 200 μg at days -1 until day 5 and then at 200 μg twice a week;

anti-B7-1 and anti-B7-2 antibodies at 250 μg at day -1 and 0 and then at 100 μg three times a week;

hCTLA4Ig at 250 μg at day -1 and 0 and then 100 μg three times a week;

Rapamycin at 1.5 mg/Kg at day -1 until day 13 and then three times a week;

mIL10 at 30 μg at day -1 and 0;

IL-12 at 1 μg at day 0; and

IL-12 at 1 μg at day 0 and then 3 times a week.

Anti-gp39 antibodies are described, e.g., PCT Patent Application No. WO 95/06666.

The percent survival of the mice is shown in Figure 12. The combination of anti-CD2 and anti-CD48 antibodies significantly protected mice againts GVHD (e.g., 75% of the mice were alive at least 70 days following injection of the alloreactive T cells). Anti-gp39 antibodies also prolonged survival of the mice (e.g., 62% of the mice were still alive at least 70 following the injection). As observed previously, CTLA4Ig mice did not have a strong effect in protecting the mice against GVHD.

Experiment 11

In this example the effect of an antibody against the adhesion molecule ICAM-2 in preventing GVHD was analyzed.

In this example, T cell depleted bone marrow, supplemented with spleen cells from BR mice, was injected into irradiated (900 TBI) B6 mice, as described above. The transplanted mice were either left untreated or treated with one of the following regimens:

anti-ICAM-2 antibody at 300 μg twice a week from day -1 to day 28;

anti-LFA-1 antibody at 300 μg twice a week from day -1 to day 28; and

anti-gp39 at 200 μg at day -1 until day 5 and then twice a week.

In another experiment, the donor cells were first treated with an antibody reactive with NK cells (NK1.1) and further with complement to delete NK cells from the donor cells.

The percent survival of the mice is shown in Figure 13. As previously described, treatment with either anti-LFA-1 or anti-gp39 prolonged survival. While not as effective as these treatments, the anti-ICAM-2 antibody also resulted in prolonged survival indicating that anti-ICAM-2 treatment can inhibit the development of GVHD.

Experiment 12

This example shows the effect of anti-IL-4 antibody on T cell responses in a mixed lymphocyte reaction.

To determine whether IL-4 production could account, at least in part, for the anti-bm12 responses in B6 $CD4^+$ T cells, an MLR culture was set up in the absence or presence of a saturating concentration (50 μg/ml) of purified anti-IL4 mAb (11B11). The MLRs were performed as described above with 1 x 10^5 B6 $CD4^+$ lymph node cells/well along with 5 x

10^5 TCD, irradiated bm12 splenic stimulators. Additional agents were also added to some MLRs for determining the effect of these agents in preventing alloresponses. The agent amounts were ≥ saturation [150μg/ml intact mAb, 50μg/ml for the other agents, except 20μg/ml for HSA]. Triplicate wells were pulsed after 4, 5 or 7 days. Day 5 was the day of peak proliferative response and therefore is used for illustration. The results are presented below:

	-----No anti-IL-4 mAb------		-----anti-IL-4 mAb-----	
Group	total cpm	%control	total cpm	%control
B6 anti-B6	2,725	-	6,190	-
B6 anti-bm12	328,007	100	150,640	46
+hCTLA4-Ig	28,312	9	37,929	12
+αB7-1	223,358	68	96,084	29
+αB7-2	128,439	39	59,285	18
+αB7-1 + αB7-2	21,164	6	51,811	16
+αCD28F(ab')$_2$	50,941	16	46,600	14
+HSA	251,802	77	123,819	38
+αCD48 + αCD2	146,334	45	67,024	20

The results indicate that IL4 protein accounts for approximately 50% of the proliferative response in B6 $CD4^+$ T cells responding to bm12 alloantigens. Thus, blocking the effect of IL-4 significantly reduces alloresponses, indicating that anti-IL-4 antibodies are likely to be useful for inhibiting GVHD either alone or in combination with other agents.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for inhibiting a response by a T cell to an antigen, comprising contacting the T cell with:

a) a first agent which inhibits a costimulatory signal in the T cell; and

b) a second agent which inhibits adhesion of the T cell to a cell which presents antigen to the T cell,

the first and second agents thereby inhibiting the response by the T cell to the antigen.

2. The method of claim 1, wherein the first agent inhibits an interaction between a receptor on the T cell and a costimulatory molecule on a cell presenting antigen to the T cell.

3. The method of claim 2, wherein the receptor on the T cell is CD28.

4. The method of claim 2, wherein the receptor on the T cell is CTLA4.

5. The method of claim 2, wherein the costimulatory molecule is B7-1 or B7-2.

6. The method of claim 2, wherein the first agent is a soluble form of CTLA4.

7. The method of claim 6, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

8. The method of claim 2, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

9. The method of claim 8, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

10. The method of claim 1, wherein the first agent acts intracellularly to inhibit generation of a costimulatory signal in the T cell.

11. The method of claim 1, wherein the second agent inhibits an interaction between an adhesion molecule on the T cell and a ligand for the adhesion molecule on a cell presenting antigen to the T cell.

12. The method of claim 11, wherein the adhesion molecule is selected from the group consisting of LFA-1, ICAM-1, ICAM-2, ICAM-3, CD49, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, CD29, CD43, CD48, VCAM-1, CD52, CD56, CD59, CD61, CD62P, LECAM-1, ELAM-1, CD44, CD103, CD104 and Thy-1.

13. The method of claim 11, wherein the second agent is an antibody, or fragment thereof, which binds either the adhesion molecule on the T cell or the ligand for the adhesion molecule on the cell that presents antigen to the T cell.

14. The method of claim 13, wherein the second agent is an anti-LFA-1 antibody, or fragment thereof.

15. The method of claim 14, wherein the anti-LFA-1 antibody is an anti-human LFA-1 monoclonal antibody, or fragment thereof.

16. The method of claim 13, wherein the second agent is an antibody selected from the group consisting of anti-gp39, anti-ICAM-1, anti-ICAM-2, and anti-CD2 together with anti-CD48.

17. A method for inhibiting graft versus host disease in a bone marrow transplant recipient, comprising contacting a first population of cells comprising donor T cells *in vitro* with:

a) a second population of cells which express recipient alloantigens;

b) a first agent which inhibits a costimulatory signal in the donor T cells; and

c) a second agent which inhibits adhesion of the donor T cells to cells which express recipient alloantigens,

the first and second agents thereby inhibiting a response by the donor T cells to the cells which express recipient alloantigens such that, upon administration of the first population of cells to the bone marrow transplant recipient, graft versus host disease in the recipient is inhibited.

18. The method of claim 17, wherein the first population of cells is selected from a group consisting of bone marrow cells, peripheral blood cells and splenocytes.

19. The method of claim 17, wherein the first agent is a soluble form of CTLA4.

20. The method of claim 19, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

21. The method of claim 17, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

22. The method of claim 21, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

23. The method of claim 17, wherein the second agent is an anti-LFA-1 antibody, or fragment thereof.

24. The method of claim 23, wherein the anti-LFA-1 antibody is an anti-human LFA-1 monoclonal antibody, or fragment thereof.

25. The method of claim 17, further comprising contacting the first population of cells with the second population of cells *in vitro* in the absence of the first and second agents, prior to contacting the first population of cells with the first and second agents *in vitro*.

26. The method of claim 17, further comprising administering the first population of cells to the recipient.

27. The method of claim 17, further comprising administering the first agent to the recipient, the second agent to the recipient, or both the first and the second agents to the recipient.

28. A method for inhibiting graft versus host disease in a donor bone marrow and donor T cell transplant recipient, comprising administering to the recipient:

a) a first agent which inhibits a costimulatory signal in the donor T cell; and

b) a second agent which inhibits adhesion of the donor T cell to a cell presenting antigen to the T cell.

29. The method of claim 28, wherein the first agent is a human CTLA4-immunoglobulin fusion protein.

30. The method of claim 2°, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

31. The method of claim 30, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

32. The method of claim 28, wherein the second agent is an anti-human LFA-1 monoclonal antibody, or fragment thereof.

33. The method of claim 28, wherein the second agent is an antibody selected from the group consisting of anti-gp39, anti-ICAM-1, anti-ICAM-2, and anti-CD2 together with anti-CD48.

34. The method of claim 28, further comprising administering donor bone marrow and donor T cells to the recipient.

35. A method for inhibiting rejection of donor allogeneic cells by a recipient of the donor allogeneic cells, comprising administering to the recipient:

a) a first agent which inhibits generation of a costimulatory signal in a recipient T cell; and

b) a second agent which inhibits adhesion of a recipient T cell to a cell presenting antigen to the T cell.

36. The method of claim 35, wherein the first agent is a soluble form of CTLA4.

37. The method of claim 36, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

38. The method of claim 35, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

39. The method of claim 38, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

40. The method of claim 35, wherein the second agent is an anti-LFA-1 antibody, or fragment thereof.

41. The method of claim 40, wherein the anti-LFA-1 antibody is an anti-human LFA-1 monoclonal antibody, or fragment thereof.

42. The method of claim 35, wherein the second agent is an antibody selected from the group consisting of anti-gp39, anti-ICAM-1, anti-ICAM-2, and anti-CD2 together with anti-CD48.

43. The method of claim 35, further comprising administering allogeneic cells to the recipient.

44. The method of claim 35, further comprising preadministering donor hematopoietic cells to the recipient prior to transplantation of the donor allogenic cells to thereby prime recipient T cells to donor alloantigens.

45. The method of claim 35, wherein the donor allogeneic cells comprise a tissue or organ.

46. The method of claim 45, wherein the tissue or organ comprises liver, kidney, heart, lung, pancreatic islets, intestine, colon, or skin.

47. The method of claim 35, further comprising contacting the donor allogenic cells with the first and seond agents *in vitro* prior to administration of the first and second agents to the recipient.

48. A method for inhibiting a response by a T cell to an antigen, comprising contacting the T cell with:

a) a first agent which inhibits a costimulatory signal in the T cell; and

b) a second agent which inhibits a proliferative signal in the T cell,

the first and second agents thereby inhibiting the response by the T cell to the antigen.

49. The method of claim 48, wherein the first agent inhibits an interaction between a receptor on the T cell a costimulatory molecule on a cell presenting antigen to the T cell.

50. The method of claim 49, wherein the receptor on the T cell is CD28.

51. The method of claim 49, wherein the receptor on the T cell is CTLA4.

52. The method of claim 49, wherein the costimulatory molecule is B7-1 or B7-2.

53. The method of claim 49, wherein the first agent is a soluble form of CTLA4.

54. The method of claim 53, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

55. The method of claim 49, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

56. The method of claim 55, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

57. The method of claim 49, wherein the first agent acts intracellularly to inhibit generation of a costimulatory signal in the T cell.

58. The method of claim 49, wherein the second agent inhibits an interaction between a receptor on the T cell and T cell growth factor.

59. The method of claim 58, wherein the T cell growth factor is selected from the group consisting of interleukin-1α, interleukin-1β, interleukin-2, interleukin-4, interleukin-6, interleukin-7, interleukin-9, interleukin-10, interleukin-12, interleukin-15, interleukin-T, and interferon-gamma.

60. The method of claim 58, wherein the second agent is an antibody which binds either to the receptor on the T cell or to the T cell growth factor.

61. The method of claim 60, wherein the second agent is an anti-interleukin-2 receptor antibody, or fragment thereof.

62. The method of claim 49, wherein the second agent acts intracellularly to inhibit generation of a proliferative signal in the T cell.

63. A method for inhibiting graft versus host disease in a bone marrow transplant recipient, comprising contacting a first population of cells comprising donor T cells *in vitro* with:

a) a second population of cells which express recipient alloantigens;

b) a first agent which inhibits a costimulatory signal in the donor T cells; and

c) a second agent which inhibits a proliferative signal in the donor T cells,

the first and second agents thereby inhibiting a response by the donor T cells to the cells which express recipient alloantigens, such that upon administration of the first population of

cells to the bone marrow transplant recipient, graft versus host disease in the recipient is inhibited.

64. The method of claim 63, wherein the first population of cells is selected from a group consisting of bone marrow cells, peripheral blood cells and splenocytes.

65. The method of claim 63, wherein the first agent is a human CTLA4-immunoglobulin fusion protein.

66. The method of claim 63, wherein the first agent is an anti-B7-1 antibody, or fragment thereof, an anti-B7-2 antibody, or fragment thereof, or both an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

67. The method of claim 66, wherein the anti-B7-1 antibody is an anti-human B7-1 monoclonal antibody and the anti-B7-2 antibody is an anti-human B7-2 monoclonal antibody.

68. The method of claim 63, wherein the second agent is an anti-interleukin-2 receptor antibody, or fragment thereof.

69. The method of claim 63, further comprising contacting the first population of cells with the second population of cells *in vitro* in the absence of the first and second agents, prior to contacting the first population of cells with the first and second agents *in vitro*.

70. The method of claim 64, further comprising administering the first population of cells to the recipient.

71. The method of claim 63, further comprising administering the first agent to the recipient, the second agent to the recipient, or both the first and the second agents to the recipient.

72. A pharmaceutical composition suitable for administration comprising an amount of a human CTLA4-immunoglobulin fusion protein and an amount of an anti-human LFA-1 antibody in a pharmaceutically acceptable carrier.

73. A pharmaceutical composition suitable for administration comprising

a) an amount of a first agent selected from the group consisting of an anti-human B7-1 monoclonal antibody, or fragment thereof, an anti-human B7-2 monoclonal antibody, or fragment thereof or both an anti-human B7-1 monoclonal antibody and an anti-human B7-2 monoclonal antibody, or fragments thereof; and

b) an amount of an anti-human LFA-1 antibody,

in a pharmaceutically acceptable carrier.

74. A pharmaceutical composition suitable for administration comprising an amount of a human CTLA4-immunoglobulin fusion protein and an anti-human interleukin-2 receptor antibody in a pharmaceutically acceptable carrier.

75. A pharmaceutical composition suitable for administration comprising

a) an amount of a first agent selected from the group consisting of an anti-human B7-1 monoclonal antibody, or fragment thereof, an anti-human B7-2 monoclonal antibody, or fragment thereof or both an anti-human B7-1 monoclonal antibody and an anti-human B7-2 monoclonal human, or fragments thereof; and

b) an amount of an anti-human interleukin-2 receptor antibody,

in a pharmaceutically acceptable carrier.

76. A method for inhibiting graft versus host disease in a donor bone marrow and donor T cell transplant recipient, comprising administering to the recipient at least one agent which inhibits a costimulatory signal in the donor T cell.

77. The method of claim 76, wherein the at least one agent is a combination of anti-B7-1 and anti-B7-2 antibody.

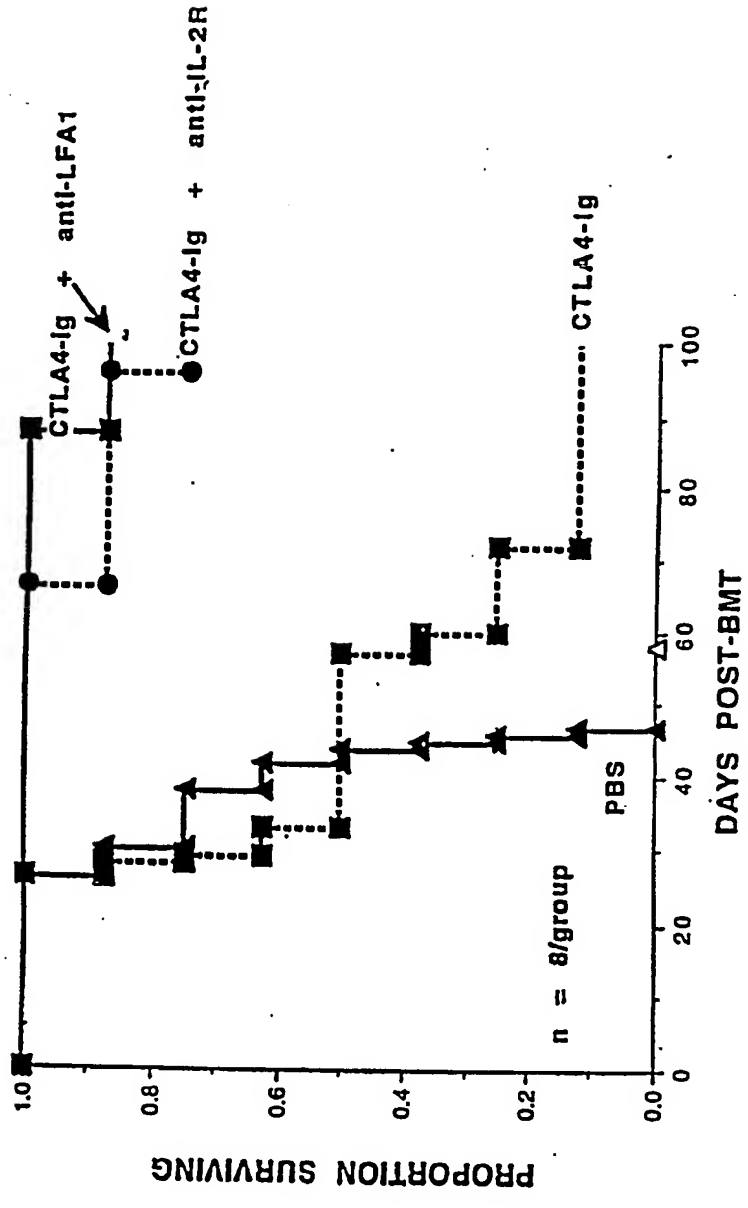

PROPORTION SURVIVING
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
DAYS POST-BMT
CTLA4-Ig + anti-LFA1
CTLA4-Ig + anti-IL-2R
CTLA4-Ig
PBS
n = 8/group

FIGURE 1

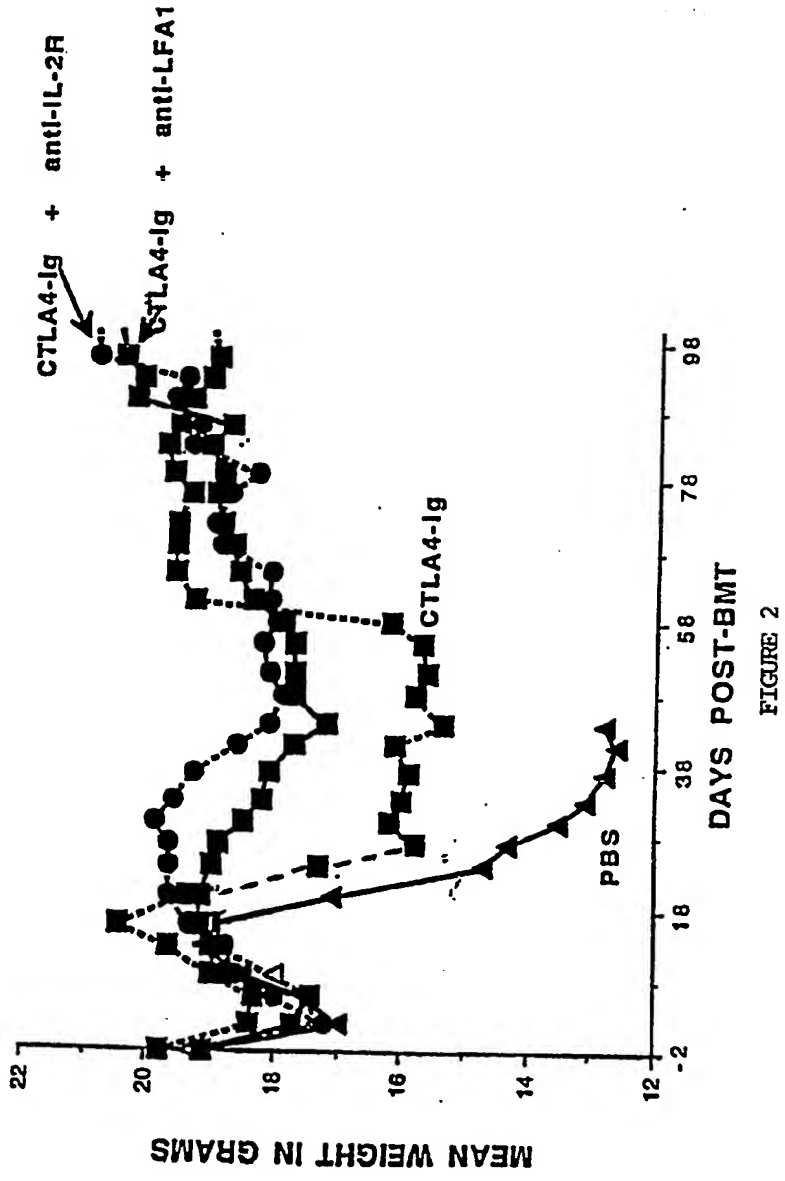

CTLA4-Ig + anti-IL-2R
CTLA4-Ig + anti-LFA1
CTLA4-Ig
PBS
MEAN WEIGHT IN GRAMS
DAYS POST-BMT
22
20
18
16
14
12
-2
18
38
58
78
98

FIGURE 2

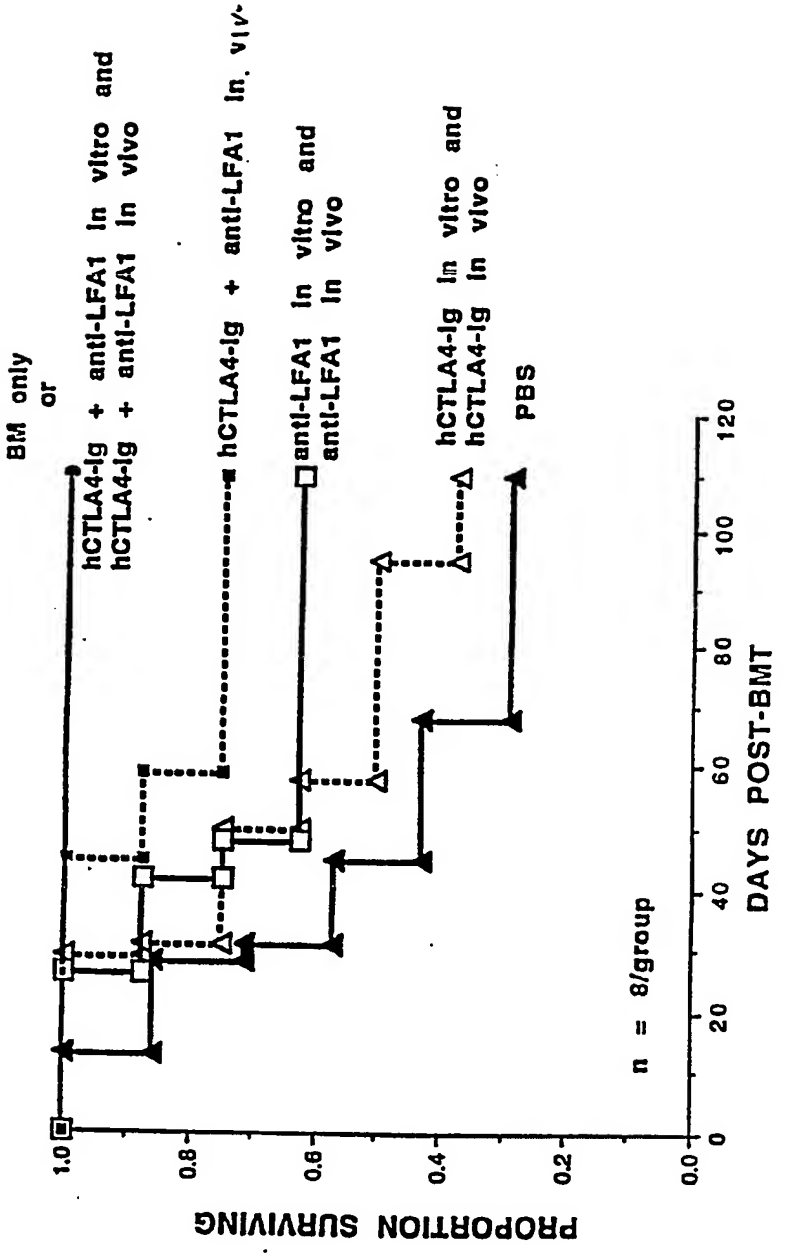

BM only
or
hCTLA4-Ig + anti-LFA1 in vitro and
hCTLA4-Ig + anti-LFA1 in vivo
hCTLA4-Ig + anti-LFA1 in vitro and
anti-LFA1 in vivo
anti-LFA1 in vitro and
anti-LFA1 in vivo
hCTLA4-Ig in vitro and
hCTLA4-Ig in vivo
PBS
n = 8/group
PROPORTION SURVIVING
DAYS POST-BMT
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
120

FIGURE 3

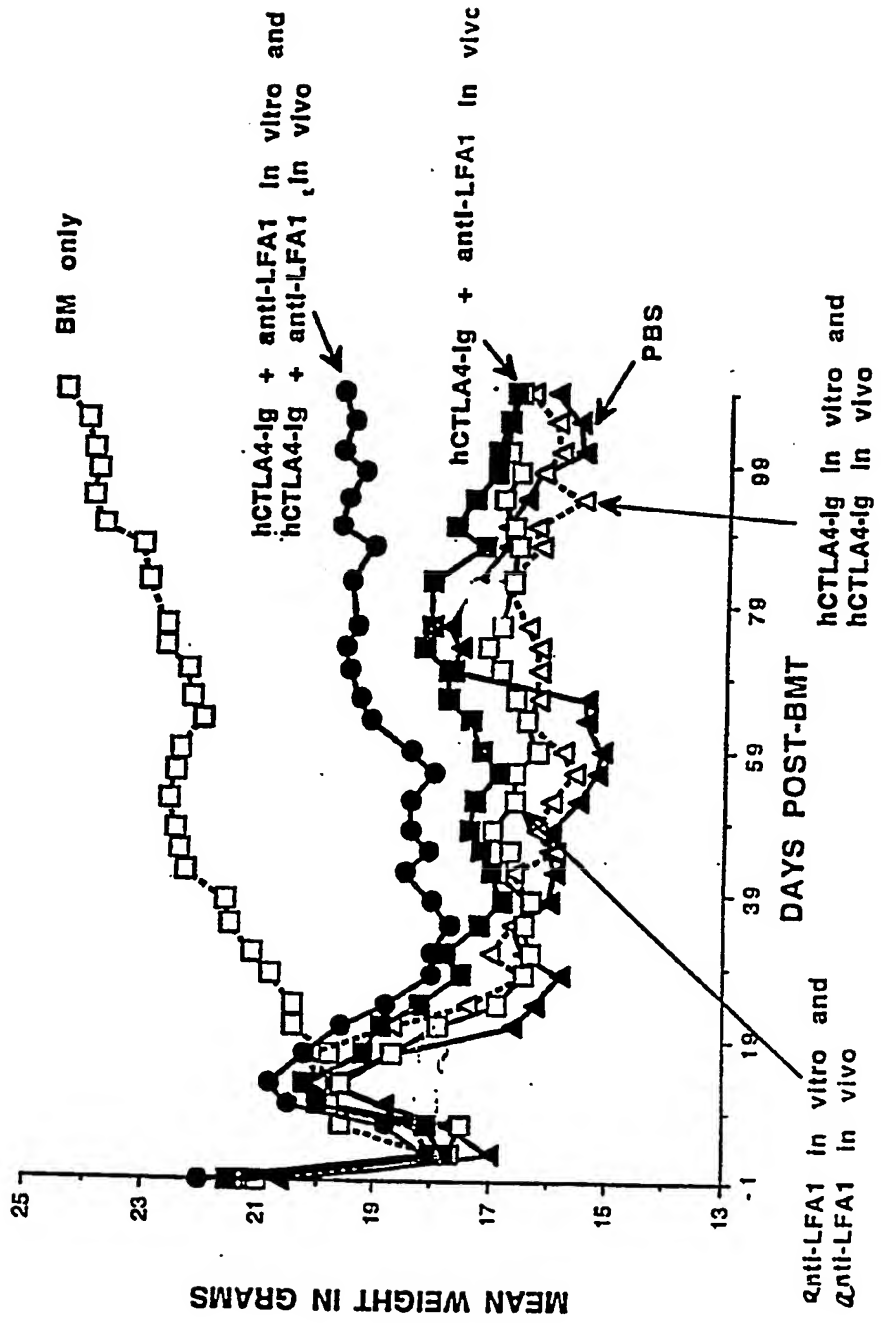

MEAN WEIGHT IN GRAMS
25
23
21
19
17
15
13
-1
19
39
59
79
99
DAYS POST-BMT
BM only
hCTLA4-Ig + anti-LFA1 in vitro and
hCTLA4-Ig + anti-LFA1 in vivo
hCTLA4-Ig + anti-LFA1 in vivo
PBS
hCTLA4-Ig in vitro and
hCTLA4-Ig in vivo
anti-LFA1 in vitro and
anti-LFA1 in vivo

FIGURE 4

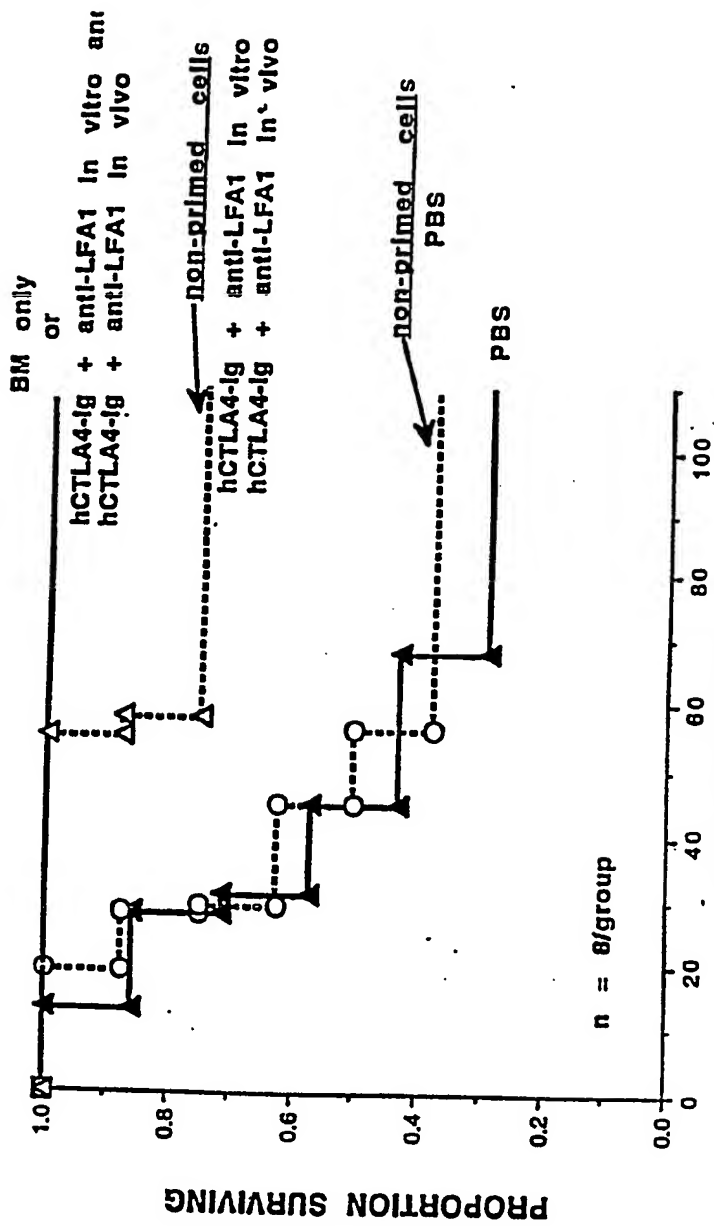

BM only
or
hCTLA4-Ig + anti-LFA1 in vitro an
hCTLA4-Ig + anti-LFA1 in vivo
non-primed cells
hCTLA4-Ig + anti-LFA1 in vitro
hCTLA4-Ig + anti-LFA1 in vivo
non-primed cells
PBS
PBS
n = 8/group
PROPORTION SURVIVING
DAYS POST-BMT
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100

FIGURE 5

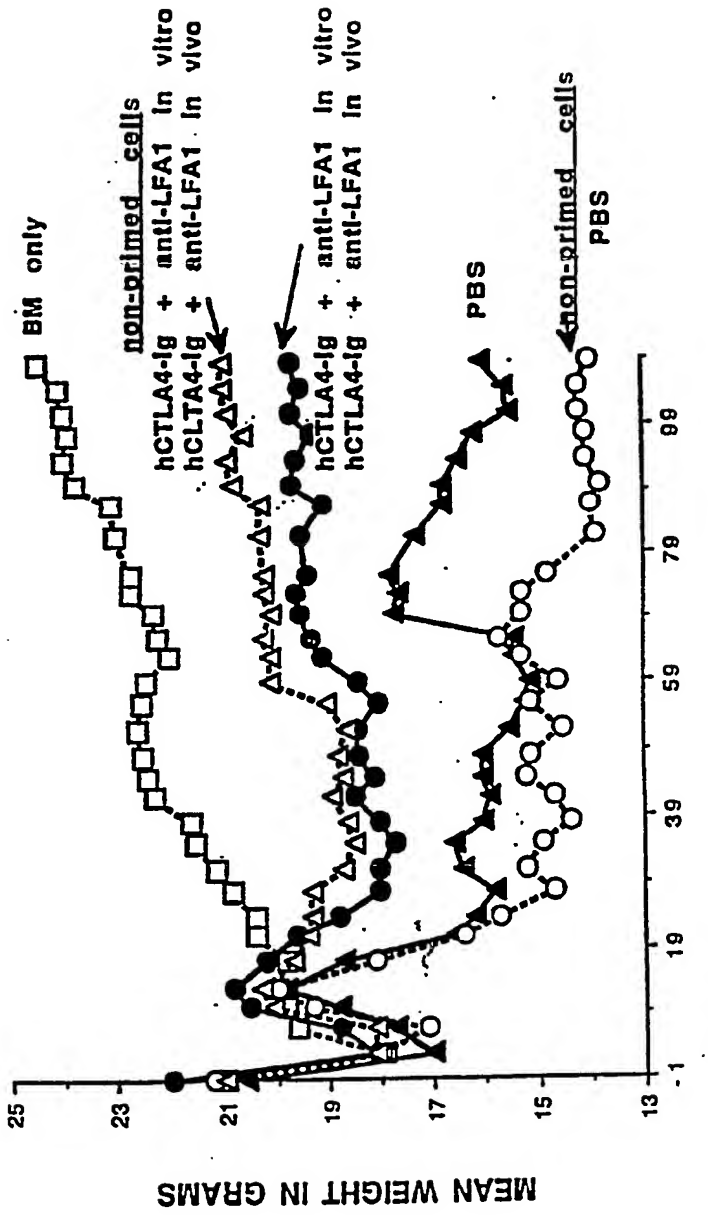

BM only
non-primed cells
hCTLA4-Ig + anti-LFA1 in vitro
hCLTA4-Ig + anti-LFA1 in vivo
hCTLA4-Ig + anti-LFA1 in vitro
hCTLA4-Ig + anti-LFA1 in vivo
PBS
non-primed cells
PBS
MEAN WEIGHT IN GRAMS
25
23
21
19
17
15
13
-1
19
39
59
79
99
DAYS POST-BMT

FIGURE 6

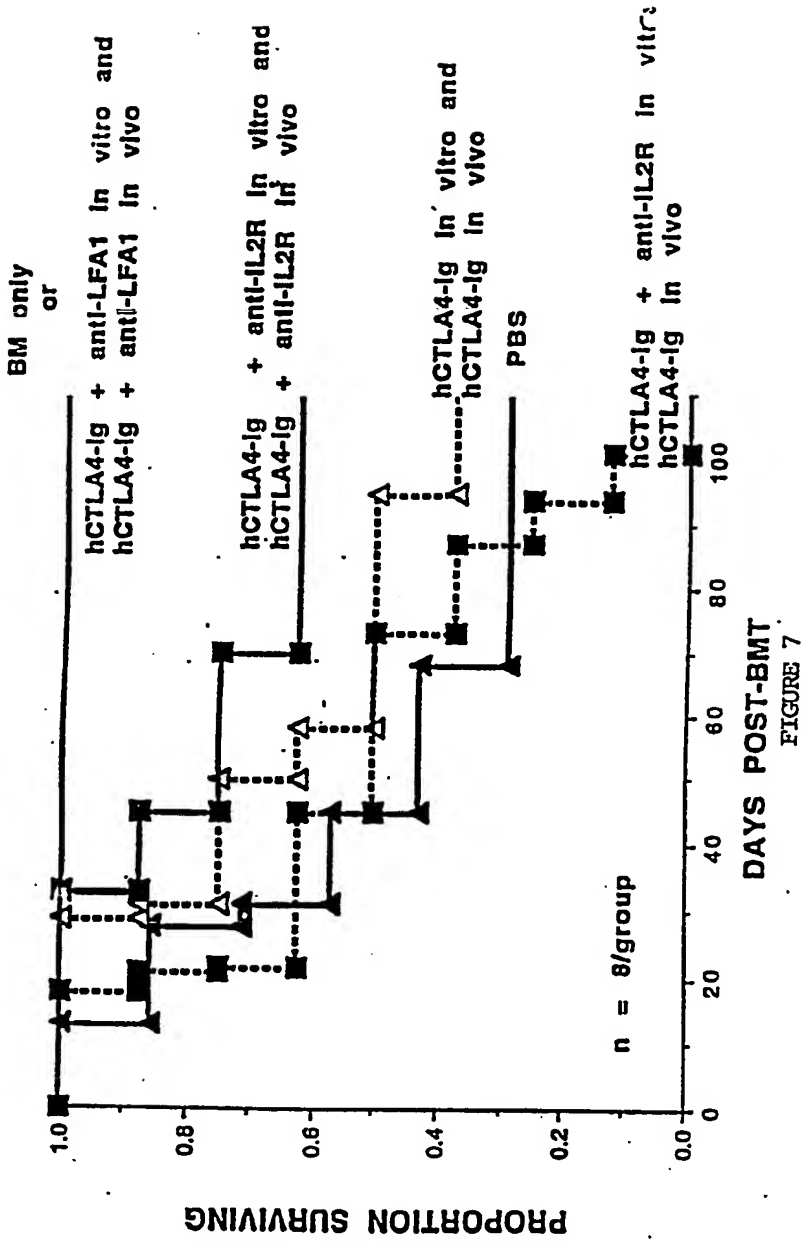

BM only
or
hCTLA4-Ig + anti-LFA1 in vitro and
hCTLA4-Ig + anti-LFA1 in vivo
hCTLA4-Ig + anti-IL2R in vitro and
hCTLA4-Ig + anti-IL2R in vivo
hCTLA4-Ig in vitro and
hCTLA4-Ig in vivo
PBS
hCTLA4-Ig + anti-IL2R in vitro
hCTLA4-Ig in vivo
n = 8/group
PROPORTION SURVIVING
DAYS POST-BMT
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100

FIGURE 7

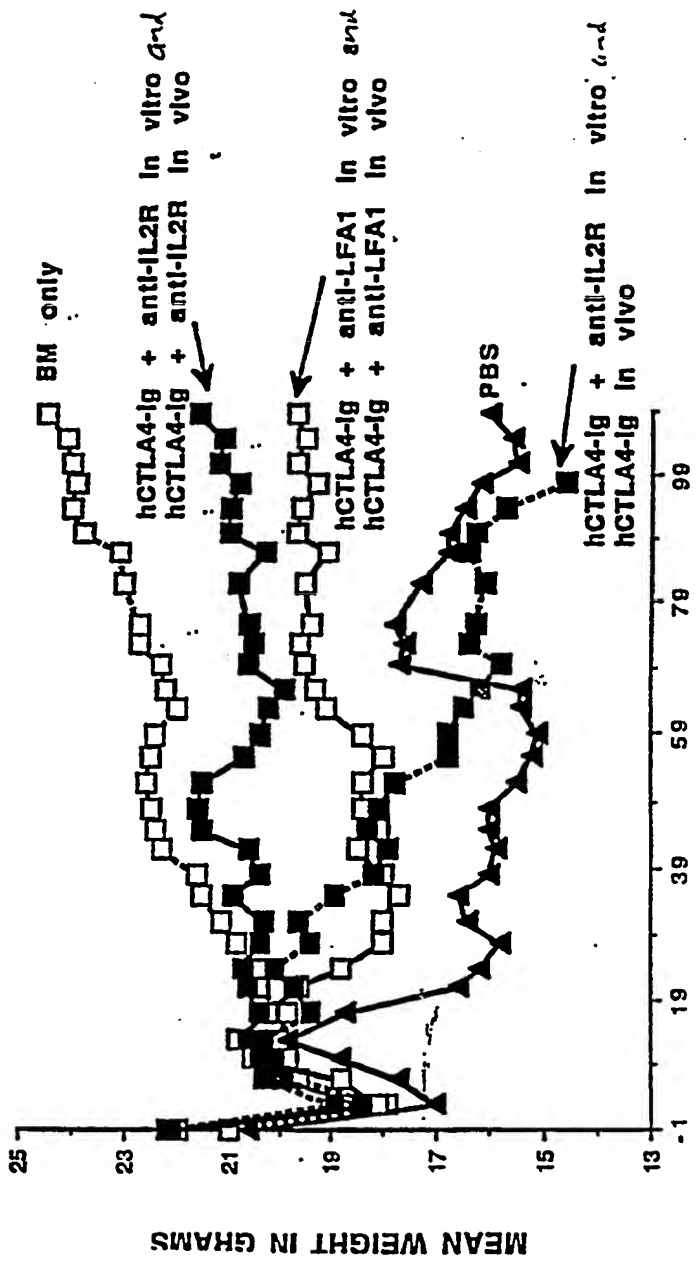

BM only
hCTLA4-Ig + anti-IL2R In vitro and
hCTLA4-Ig + anti-IL2R In vivo
hCTLA4-Ig + anti-LFA1 In vitro and
hCTLA4-Ig + anti-LFA1 In vivo
PBS
hCTLA4-Ig + anti-IL2R In vitro and
hCTLA4-Ig In vivo
MEAN WEIGHT IN GRAMS
25
23
21
19
17
15
13
-1
19
39
59
79
99
DAYS POST-BMT

FIGURE 8

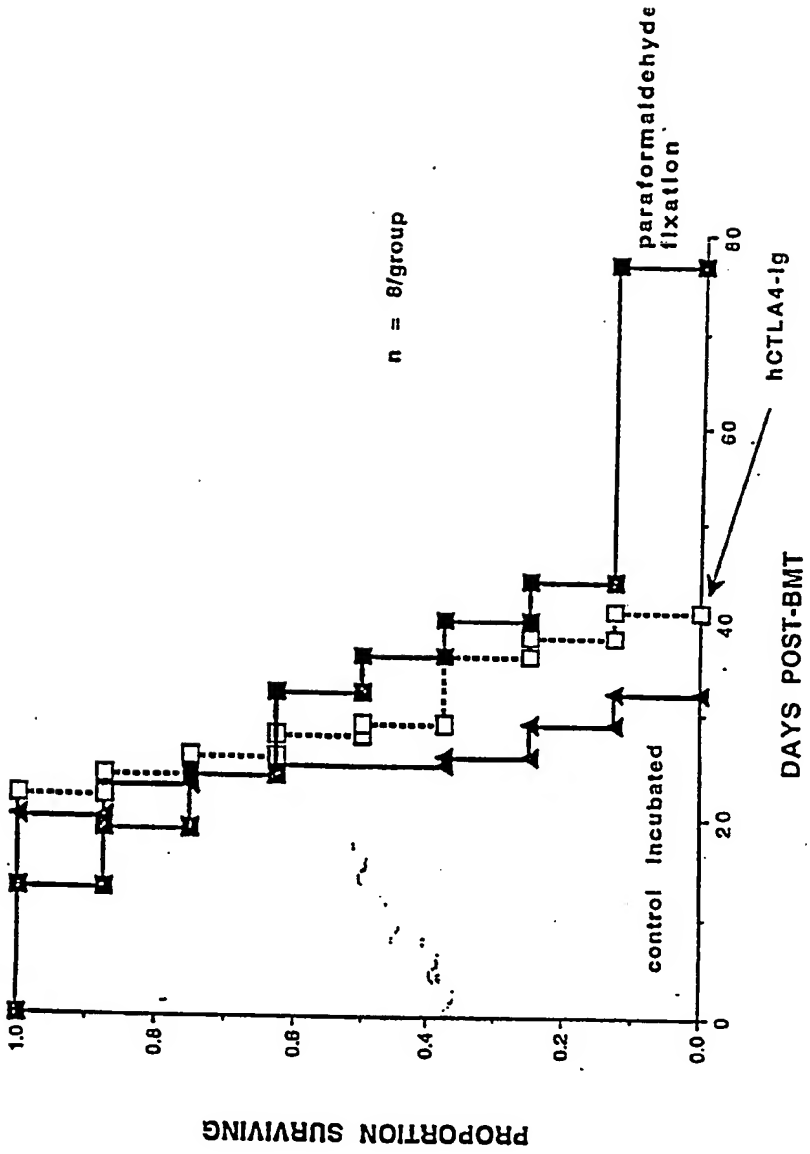

PROPORTION SURVIVING
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
DAYS POST-BMT
n = 8/group
paraformaldehyde fixation
hCTLA4-Ig
control Incubated

FIGURE 9

FIG. 10
Anti-B7-1 and Anti B7-2

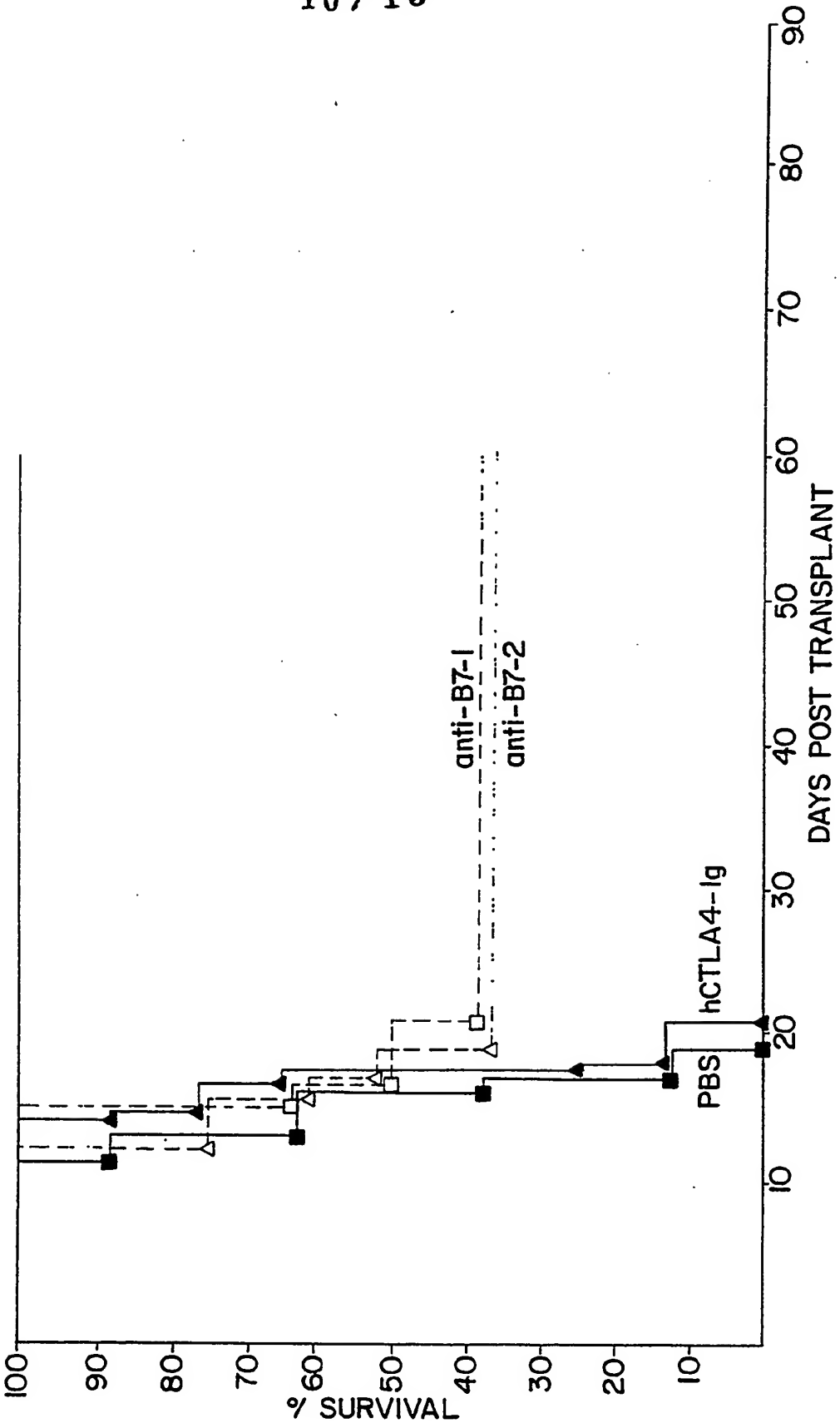


FIG. 11

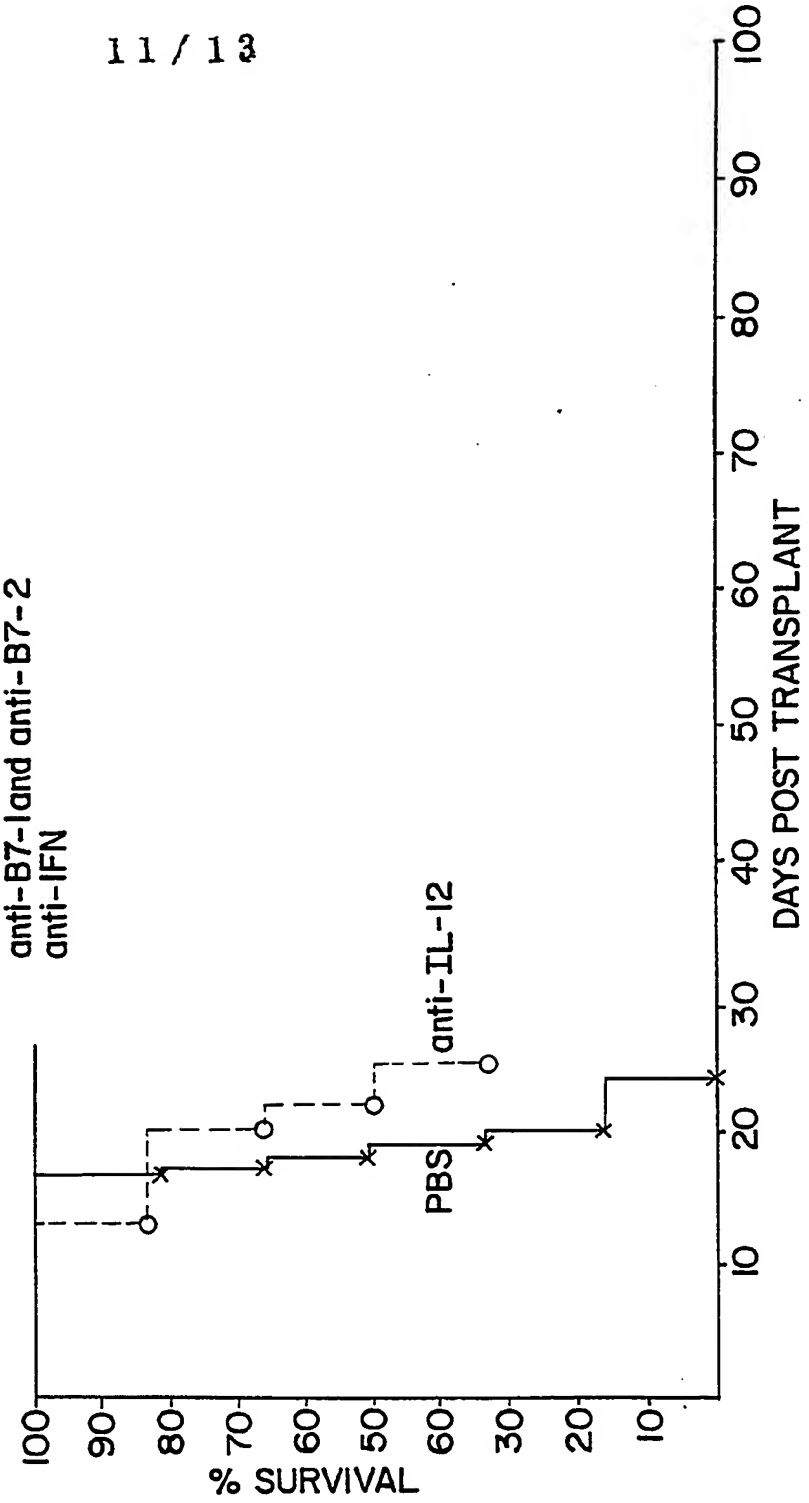

anti-B7-1 and anti-B7-2
anti-IFN
anti-IL-12
PBS
% SURVIVAL
DAYS POST TRANSPLANT
100
90
80
70
60
50
40
30
20
10

FIG. 12

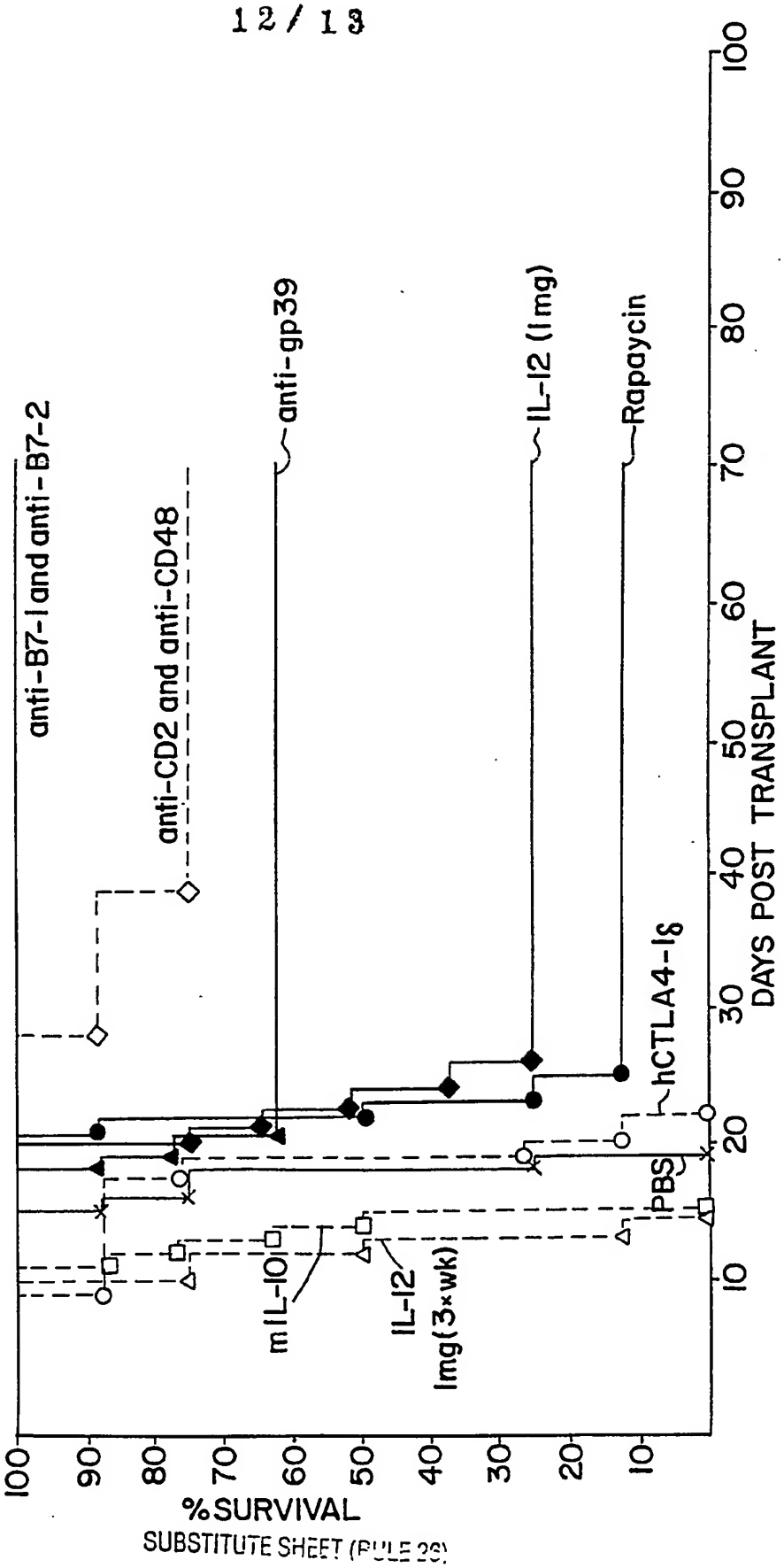
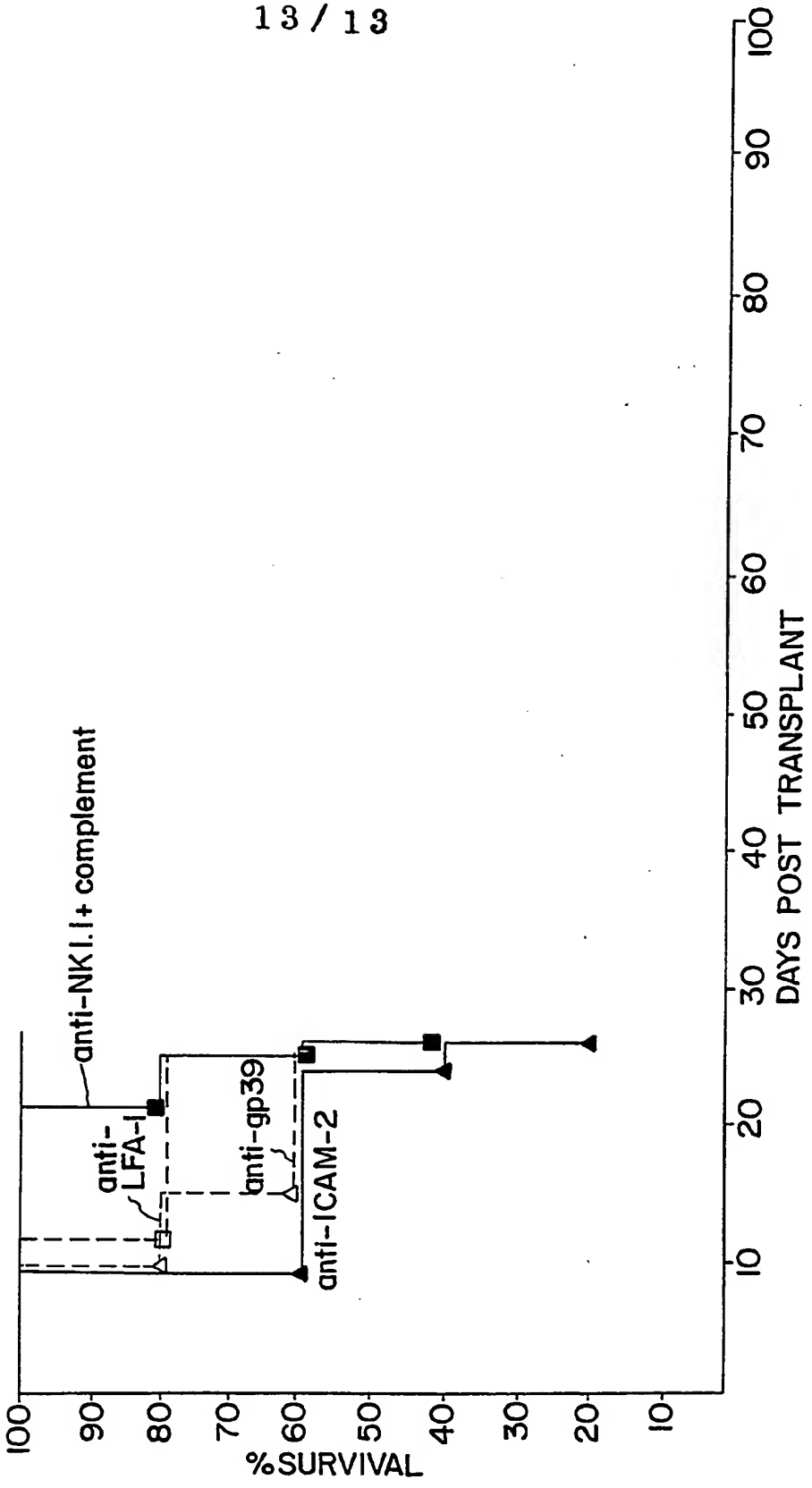

anti-B7-1 and anti-B7-2
anti-CD2 and anti-CD48
anti-gp39
IL-12 (1mg)
Rapaycin
hCTLA4-Iδ
PBS
mIL-10
IL-12
1mg(3×wk)
%SURVIVAL
DAYS POST TRANSPLANT
100
90
80
70
60
50
40
30
20
10

FIG. 13


anti-NK1.1+ complement
anti-LFA-1
anti-gp39
anti-ICAM-2
%SURVIVAL
DAYS POST TRANSPLANT
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100